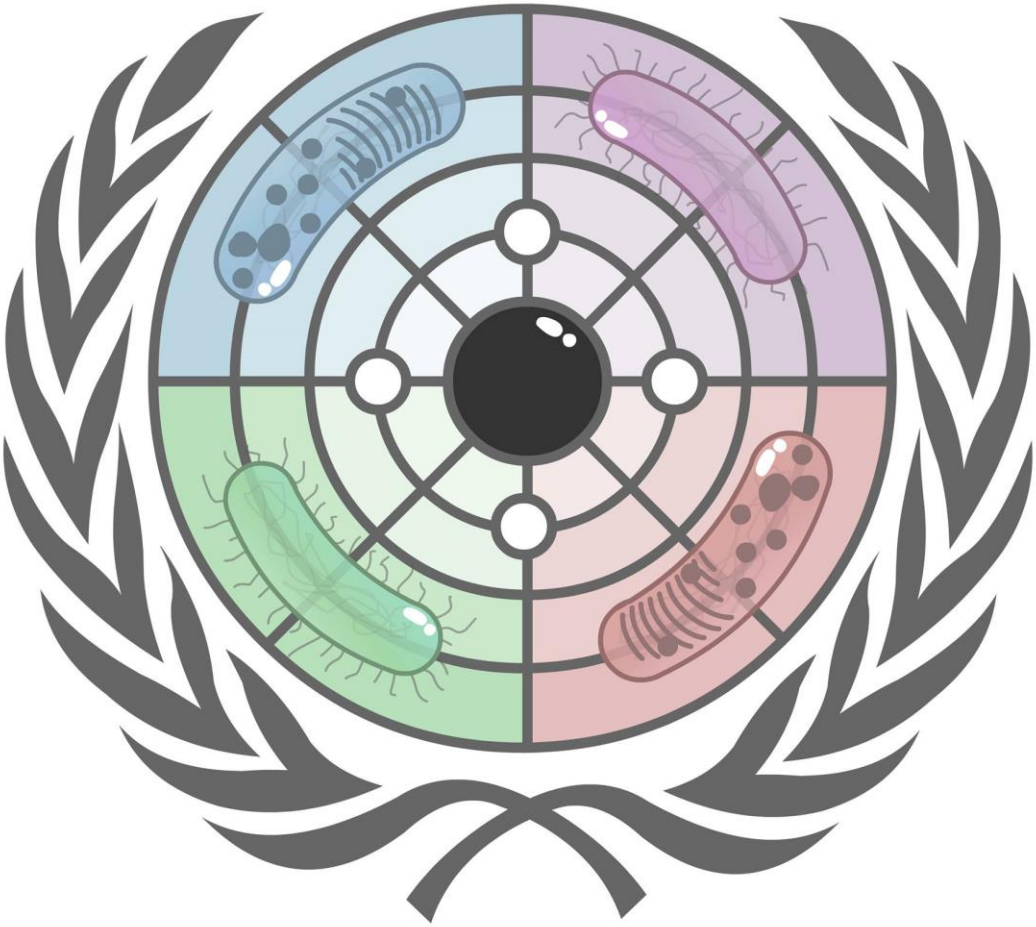
The methanotrophic interactome: Microbial partnerships for sustainable methane cycling

ir. Frederiek-Maarten Kerckhof



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The methanotrophic *interactome*: Microbial partnerships for sustainable methane cycling

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Opgedragen aan:

Dr. Jozef Kerckhof

NOTATION INDEX

A	Adenin
BLAST	Basic Local Alignment Search Tool
Bp	Base pares
C	Cytosin
CFU	Colony Forming Unit
Co	Community Organization
DGGE	Denaturating Gradient Gel Electrophoresis
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNMS	diluted Nitrate Mineral Salts
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediamine Tetracetic Acid
EPS	Extracellular polymeric substances
FADH	Formaldehyde dehydrogenase
FDH	Formate dehydrogenase
FID	Flame Ionization Detector
FWER	Family-wise error rate
G	Guanin
G+	Gram-positive
G-	Gram-negative
GC	Gas Chromatography
GHG	Greenhouse gas
IPCC	Intergovernmental Panel on Climate Change
LCA	Last Common Ancestor
LOD	Limit of detection
LOQ	Limit of quantification
MDH	Methanol dehydrogenase
MOB	Methanotrophic bacteria, methanotrophs, aerobic MOB

MOR	Methane oxidation rate
MRM	Microbial Resource Management
N	Total count
NA	Nutrient Agar
NMS	Nitrate Mineral Salts
Nt	Nucleotide
NCBI	National Center for Biotechnology Information
OD	Optical Density
OTU	Operational Taxonomic Unit
Ppmv	Volumetric parts per million
PC	Principal Component
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
pMMO	Particulate Methane Mono-oxygenase
RDP	Ribosomal Database Project
Rr	Range-weighted Richness
RNA	Ribonucleic Acid
SCP	Single-cell protein
SG	SYBR Green
SIP	Stable isotope probing
sMMO	Soluble Methane Mono-oxygenase
SRA	Sequence Read Archive
T	Thymin
TAE	Tris-Acetate-EDTA
TSA	Trypticase Soy Agar
QQ	Quantile-Quantile plot
qPCR	Quantitative (real-time) polymerase chain reaction

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Introduction

CHAPTER 1 INTRODUCTION

Chapter partially redrafted after:

- Ho, A., Kerckhof, F.-M., Lüke C., Reim, A., Krause, S., Boon, N. Bodelier, P.L.E. (2013) Conceptualizing functional traits and ecological characteristics of methane-oxidizing bacteria as life strategies. *Environmental Microbiology Reports*, 5(3), 335-345.

1. Global methane cycling: sources and sinks

This thesis will discuss the involvement of microorganisms in the biological aerobic oxidation of methane (CH_4). Methane is the simplest of all alkanes consisting of a central carbon (C) atom surrounded by four hydrogen (H) atoms. Aerobic oxidation of gaseous CH_4 with 2 mole oxygen (O_2) per mole CH_4 to one mole of carbon dioxide (CO_2) and 2 moles of water (H_2O) yields 842.3 kJ mol^{-1} K (ΔG_r^0) .

1.1. Methane as a greenhouse gas (GHG)

Without the greenhouse effect, life on earth (as we know it) would probably not be possible, as half of the solar energy reaching earth would reflect of the surface and be lost to space. Greenhouse gasses in the troposphere cover the earth with a "heat blanket" (Kandel and Viollier 2005) resulting from their absorption of reflected heat and re-radiation to the lower troposphere which finally contributes to an increase in earth surface temperatures (Casper 2010). After water vapour and CO_2, methane (CH_4) is the third most important greenhouse gas, and the majority (ca. 60%) of its emission originated from anthropogenic sources (Kirschke, Bousquet *et al.* 2013). Atmospheric CH_4 concentrations increased by 150% since the pre-industrial era (722 ppb in 1750 to 1803 ppb in 2011). Although atmospheric CH_4 concentrations stabilized in the late 1990s, CH_4 concentrations have been increasing again

since 2007 (Nisbet, Dlugokencky *et al.* 2014). Recently, the Intergovernmental Panel on Climate Change (IPCC) increased the global warming potential of methane from 25 to 34, using a time horizon of 100 years with inclusion of climate-carbon feedback (Myhre, Shindell *et al.* 2013). Hence, since CH_4 is a more potent greenhouse gas than CO_2, removal of CH_4 to CO_2 by methane oxidizing bacteria can be considered as a net greenhouse gas sink. A reduction of CH_4 emissions with only 6% (22 Mton CH_4 yr^{-1}, 2004 estimate) would suffice to balance the global CH_4 budget (Jardine, Boardman *et al.* 2004). Given the importance of CH_4 as the third most important greenhouse gas, accounting for about 20 % of global warming (Yusuf, Noor *et al.* 2012), the great anthropogenic contribution to its emissions and the relatively small emission reduction required to balance the atmospheric budget, an in-depth understanding of biological methane removal is of great merit.

1.2. Methane sources

The main biological source of CH_4 lies within the anaerobic degradation of organic matter through a process called methanogenesis, which requires an intricate network of cross-feeding (syntrophic) organisms to supply methanogenic Archaea with H_2 and CO_2 or acetate in absence of O_2 (Angenent, Karim *et al.* 2004; Cabezas, de Araujo *et al.* 2015) enabling the production of biogas with a typical composition of 40-70% (v/v) of CH_4, 30-60% (v/v) CO_2 and 1-5% (v/v) other gasses (e.g. NH_3, H_2S and H_2, Demirel, Scherer *et al.* (2010)). These methanogenic Archaea are the most important methane source and account for approximately 70% of the total emission budget of more than 600 Tg per year (Dalsøren, Myhre *et al.* 2016). Most of these biogenic CH_4 emissions (63% of 347 Tg CH_4 yr^{-1}) originate from wetlands (Figure 1-1) which account for the main inter-annual variability of emissions. However, process-based models still fail to accurately describe wetland emissions. Apart from biogenic sources, also thermogenic sources (from the slow transformation of organic matter into fossil fuels) and pyrogenic sources (from biomass and biofuel burning) contribute to the global CH_4 budget (Neef, van Weele *et al.* 2010). The fossil component of emissions could be higher than previously estimated and amount up to 30% of total emissions due to natural geological leaks but also due to leaks in the fossil fuel industry (for instance the recent southern California gas leak in Aliso Canyon, emitting 0.1Tg CH_4 from October 2015 to February 2016). Overall, a better understanding of anthropogenic emission sources (such as livestock and oil and gas sectors) is required (Ciais, Sabine *et al.* 2014; Turner, Jacob *et al.* 2016). Another important climate-carbon feedback may reside in thawing of permafrost and CH_4 - hydrates however the

extent of possible emissions is still largely unknown, and in the current decade they represent only very small emissions (2-9 Tg CH_4 yr^{-1}). The amount of natural emissions other than wetlands is still very uncertain. For instance emissions by ebullition from shallow lakes could be significantly higher than estimated (Aben 2016) and although CH_4 emissions under aerobic conditions by plants were considered unlikely (Ciais, Sabine *et al.* 2014) some recent studies showed that emissions from the trunks of living trees (rather than the canopy and soil) may be a widespread and not previously considered source of atmospheric CH_4 emission (Pangala, Moore *et al.* 2013; Pangala, Hornibrook *et al.* 2015; Wang, Gu *et al.* 2016). Sporadic geological events such as the Mount Pinatubo volcanic eruption in 1991 (Banda, Krol *et al.* 2013) may offset the atmospheric CH_4 balances (not necessarily through direct emission of CH_4 though rather by a decrease in available atmospheric OH due to backscattering by volcanic stratospheric aerosols which reduce UV radiation in the troposphere; Dalsøren, Myhre *et al.* (2016)) and need to be corrected for in bottom-up emission models (Schaefer, Fletcher *et al.* 2016).

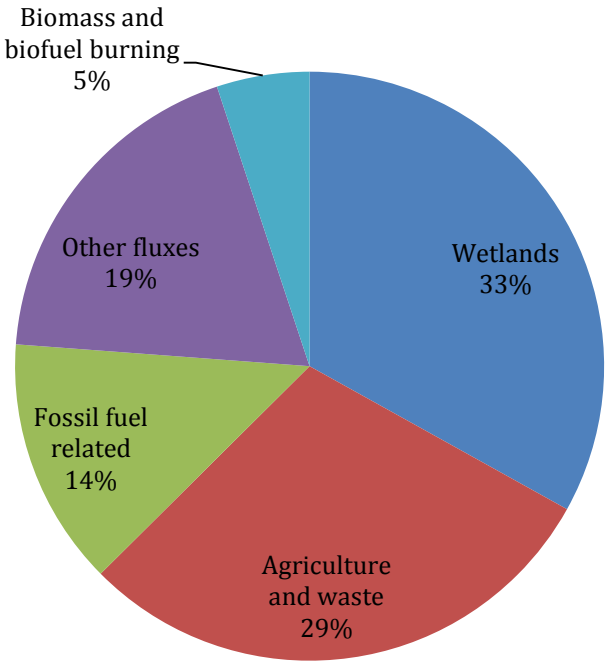


Figure 1-1. Distribution of CH_4 emission over different sources (Ciais, Sabine et al. 2014). *Average of bottom-up estimates for each source for the decade 2000-2009. Wetlands refer to natural wetland (wet soil, swamps, bogs & peatlands) emissions, agriculture and waste encompass rice cultivation, ruminant livestock and waste (e.g. landfills). Fossil fuel related emissions can be attributed to emissions caused by leakages from extraction and use or pyrogenic sources such as incomplete burning. Other fluxes encompass (among others) geological sources (marine & terrestrial seepages, geothermal vents and mud volcanoes) , termite- and freshwater emissions.*

1.3. Methane sinks and reservoirs

Atmospheric CH_4 is mainly removed by tropospheric and stratospheric oxidation with OH radicals, leading to an atmospheric lifetime of 9.1±0.9 years (Prather, Holmes *et al.* 2012). Some other small sinks are still debated (e.g. reactions with chlorine in marine boundary layer and stratospheric reaction with Cl and $O(^1D)$). Finally, microbial atmospheric methane oxidation in aerobic upland soils is estimated to account for about 7.5 % (9-47 Tg CH_4 yr^{-1}) of total annual methane removal, thus making it the most important biotic methane sink (Figure 1-2; Ciais, Sabine *et al.* (2014)). Moreover, MOB can attenuate CH_4 emissions in diverse environments which are considered net sources of CH_4 (Conrad 2009; Knief 2015). Both (intra-)aerobic and anaerobic modes of methane oxidation by bacteria and archaeal-bacterial associations have been observed, though aerobic methanotrophic bacteria are considered the main microbial methane sink (Ho, Kerckhof *et al.* 2013; Hoefman, van der Ha *et al.* 2014). However, in terms of rates it important to consider that anaerobic oxidation of methane (AOM, section 2.2.2 of this chapter) at the sulfate/methane transition zone is attenuating the greatest amount of CH_4 emissions (up to 80-90% of the CH_4 from marine systems is attenuated this way, Hinrichs and Boetius (2002)) while strictly speaking this is not a post-emission sink such as the upland soils it is a very important mechanism attenuating global CH_4 emissions (Knittel and Boetius 2009).

Aside from sources (section 1.2) and sinks of CH_4, natural reservoirs for CH_4 exist (Figure 1-2). Methane hydrates in sediments at the ocean floor and in permafrost account for $2*10^6$ to $8*10^6$ Tg CH_4 and approximately $530*10^3$ Tg CH_4 respectively (Ciais, Sabine *et al.* 2014). As a result of the rising temperature since pre-industrial times CH_4 from permafrost hydrates may be released to the atmosphere (Schuur, Abbott *et al.* 2013), however there is only limited confidence on the magnitude of these emissions (Ciais, Sabine *et al.* 2014).

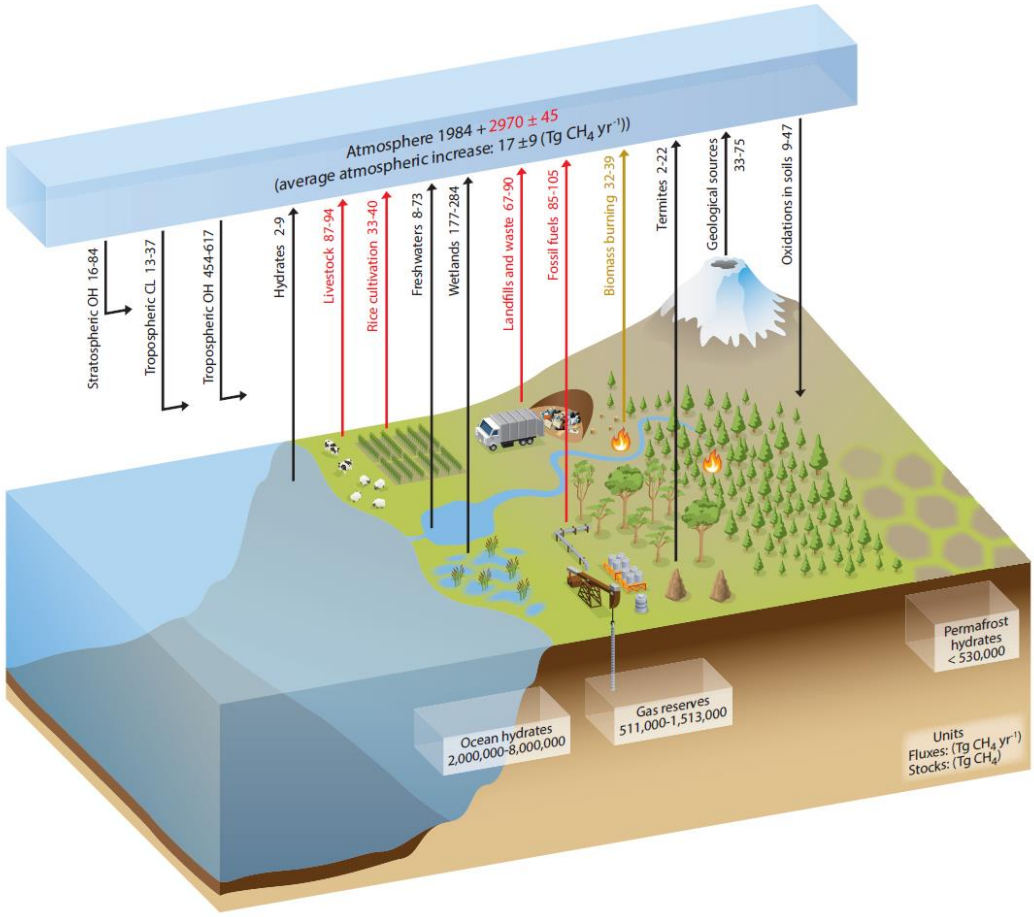


Figure 1-2. Schematic of the global methane cycle (Ciais, Sabine et al. 2014). *Numbers represent annual fluxes in Tg CH_4 yr^{-1}(2000-2009) and reservoirs (the atmosphere and three geological reservoirs) in Tg CH_4. Black arrows denote non- (directly) anthropogenic fluxes (since 1750) while red arrows denote anthropogenic fluxes. While light brown denotes a combined natural and anthropogenic flux. The atmospheric reservoir concentration (in 2011) consists of the sum of anthropogenic (red, between 1750 and 2011) and pre-industrial inventory (in black).*

2. Methanotrophic bacteria

Given their importance as methane sink (section 1.3), one strategy for methane mitigation, as well as recovery of the energy and carbon from CH_4, could rely on the use of methanotrophic bacteria (methanotrophs, MOB). MOB are a subset of the physiological group of methylotrophic bacteria known to use reduced carbon substrates with no carbon bonds (C1 compounds) for anabolism and catabolism (Lidstrom 2006; Chistoserdova, Kalyuzhnaya *et al.* 2009). Within this group they are characterized by their unique ability to use CH_4 as a sole carbon and energy source (Hanson and Hanson 1996; Bowman 2006).

2.1. Phylogeny, taxonomy and classification

The majority of the cultured aerobic MOB can be classified within the clades of Alpha- and Gammaproteobacteria (Semrau, DiSpirito *et al.* 2010; Knief 2015). These lineages can be differentiated based on their physiology, which may reflect their adopted life strategies (Ho, Kerckhof *et al.* 2013). However, a large proportion of environmental MOB sequences has no cultured representatives (Knief 2015) and recent isolates have expanded the taxonomy of MOB to include the *candidatus* NC10 phylum (Ettwig, van Alen *et al.* 2009) with the intra-aerobic nitrite-dependent anaerobic methane oxidizer candidatus *Methylomirabilis oxyfera* (Wu, Ettwig *et al.* 2011) as well as the phylum Verrucomicrobia (Dedysh 2009) with representatives *Methylacidimicrobium* (van Teeseling, Pol *et al.* 2014) and *Methylacidiphilum* (Hou, Makarova *et al.* 2008; Anvar, Frank *et al.* 2014). Additionally a new family was proposed within the Methylococcales, the *Methylothermaceae* (Hirayama, Abe *et al.* 2014). Although a surprisingly high number of environmental *pmoA* sequences could be linked with closely affiliated cultured representatives, still a large fraction of the MOB diversity is not yet isolated (Knief 2015). Hence, the taxonomy of aerobic MOB as outlined in Figure 1-3 will likely still expand upon increased isolation efforts.

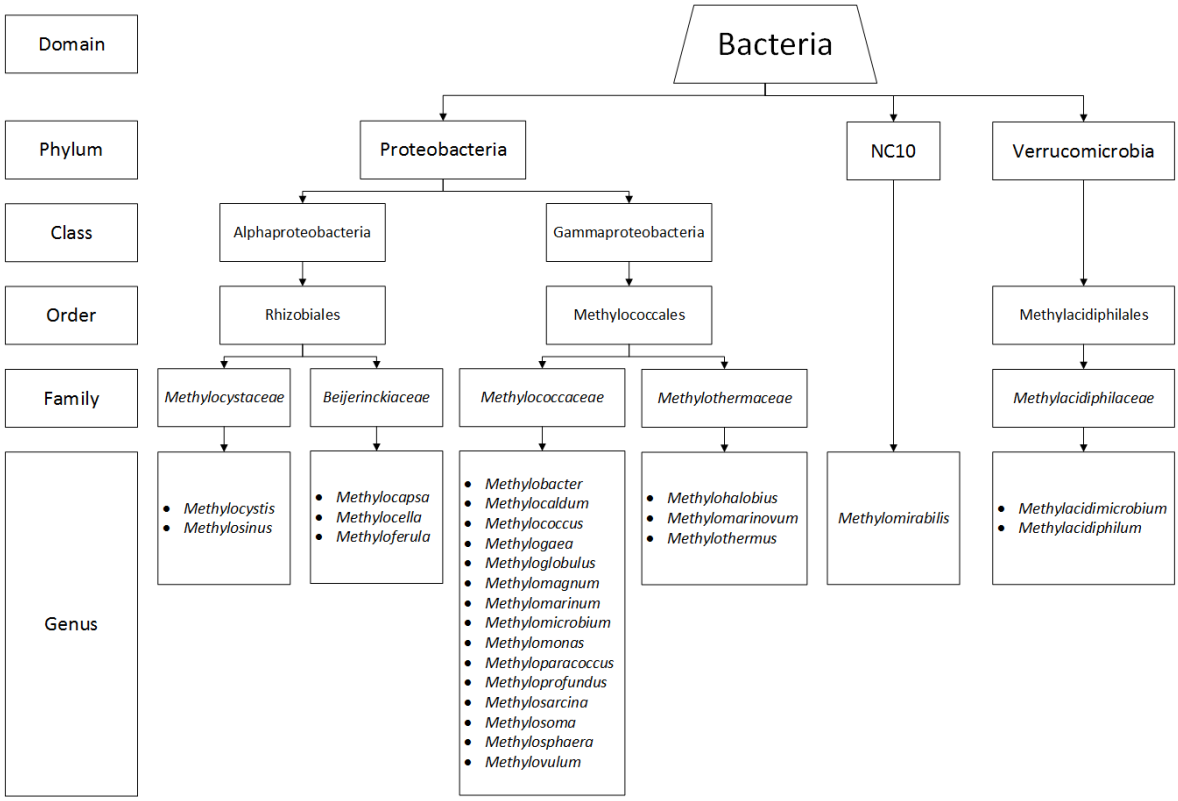


Figure 1-3. Taxonomy of isolated and cultivated aerobic MOB. *Updated from Dedysh (2009) with more recent isolates (Geymonat, Ferrando et al. 2011; Iguchi, Yurimoto et al. 2011; Hirayama, Fuse et al. 2013; Deutzmann, Hoppert et al. 2014; Hirayama, Abe et al. 2014; Hoefman, van der Ha et al. 2014; van Teeseling, Pol et al. 2014; Knief 2015; Tavormina, Hatzenpichler et al. 2015).* ***Figure on previous page.***

Traditionally, a classification of proteobacterial methanotrophs based on several physiological, (chemo)taxonomic (i.e. major cellular fatty acids) and morphological characteristics (e.g. arrangement of intracyctoplasmatic membranes) into type I, type II and type X MOB was believed to adequately describe the proteobacterial MOB diversity (Hanson and Hanson 1996). The major distinction was the main carbon assimilation mechanism (section 2.2), being the ribulose monophosphate (RuMP) pathway for type I and serine cycle for type II MOB. Type X MOB were distinguished from type I MOB based upon higher optimum growth temperatures, presence of low levels of serine cycle enzymes (while mainly relying on the RuMP pathway of formaldehyde assimilation, Nazaries, Murrell *et al.* (2013)) presence of ribulose-1,5-bisphosphate carboxylase and differing nitrogen fixation capability (Hanson and Hanson 1996; Bowman 2006). Type I and type X MOB are also sometimes referred to as type Ia and type Ib MOB (Bodrossy, Stralis-Pavese *et al.* 2006; Lüke and Frenzel 2011). However, based upon the characterization of new isolates belonging to several new genera and species the distinction between the type I(a/b) and type II MOB has become increasingly less clear, except for the difference in major carbon assimilation pathway (Knief 2015). Hence, type I and type II MOB should be considered synonyms for MOB in taxonomically classified to Gamma- and Alphaproteobacteria respectively. An operational classification into subgroups of type I (a,b,c) and type II (a, b) MOB was proposed by Knief (2015) with verrucomicrobial MOB refered to as type III. This classification was consistent with 16S rRNA gene phylogeny (Knief (2015) and Figure 1-4).

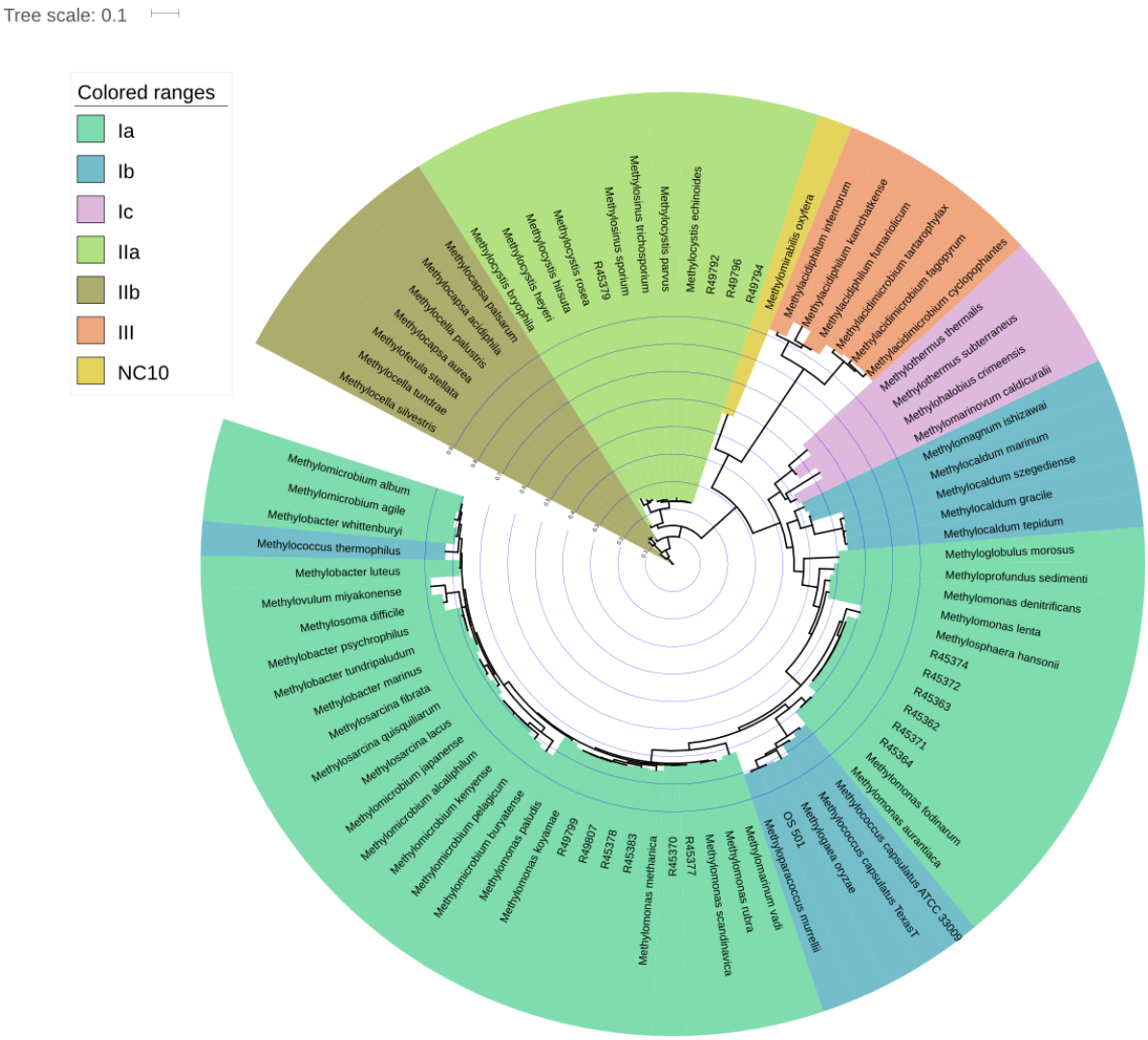


Figure 1-4. Phylogenetic tree of the 16S rRNA gene of methanotrophic type strains and some isolates used in this and earlier work (Hoefman, van der Ha et al. 2014). *RAxML version 8.2.6 (Stamatakis 2014) was used to perform maximum likelihood tree building under the generalized time reversible model (GTR) with the Gamma model of rate heterogeneity (GAMMA). The best tree of 1000 bootstrap iterations is displayed (final ML optimization likelihood -13913.80). Using mothur 1.35.1 (Schloss, Westcott et al. 2009), a NAST alignment of full-length 16S rRNA gene sequences (obtained with Straininfo.net's SeqRank workflow) with the mothur-recreated SILVA seed alignment (release 123) was trimmed to the same alignment space resulting in 695 distinct alignment positions. The parsimony random number seed was set to 12345 and the random seed for rapid bootstrapping was 123. Colored ranges correspond to MOB types according to Knief (2015). Tree visualisation was performed using iTOL version 3 (Letunic and Bork 2011). Scale=0.1 mean number of substitutions per site.*

2.2. Physiology

2.2.1. Aerobic methylotrophs and methanotrophs

All methylotrophs (including the methanotrophs) share a common general scheme for assimilation of C1 substrates (Figure 1-5): (1) oxidation of a primary methylated substrate to formaldehyde (CH_2O) or methylene-tetrahydrofolate (CH_2=H_4F), (2) oxidation of the resulting formaldehyde (or methylene-H_4F) to CO_2 and (3) assimilation of a C1 unit through either: formaldehyde with the ribulose monophosphate (RuMP) pathway, CO_2 through the Calvin-Benson-Bassham (CBB) cycle or methylene-H_4F and CO_2 using the serine cycle.

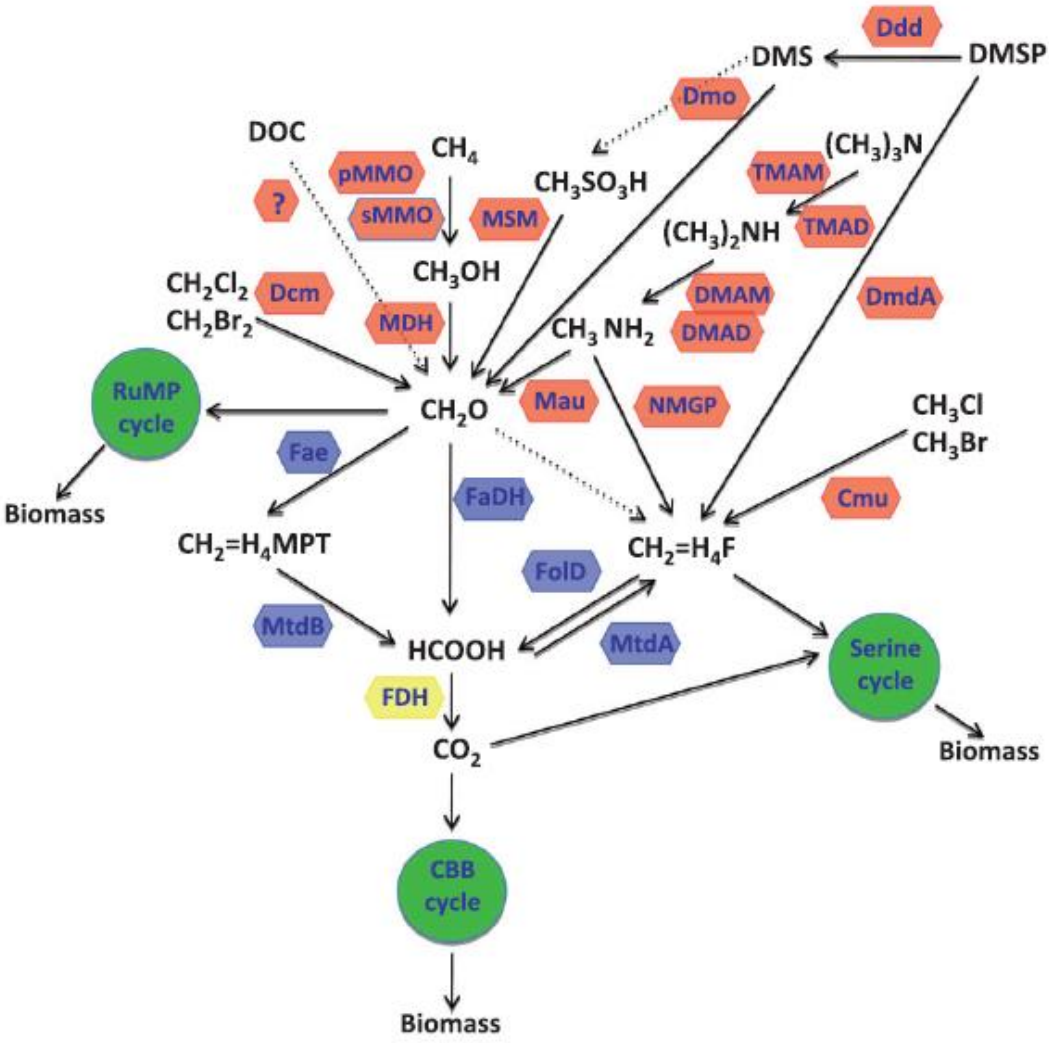


Figure 1-5. Major substrates and intermediates in methylotrophic metabolism (Chistoserdova 2011). *Primary oxidation (or demethylation/dehalogenation) modules are shown in red, formaldehyde (methyl-H_4F) handling modules in blue, formate dehydrogenase in yellow and assimilation modules in green. For more details on the abbreviations we refer the reader to (Chistoserdova 2011).*

Methane oxidation to methanol

Unique to the MOB physiology are methane mono-oxygenases (MMO) which enable the MOB to perform the difficult and rate-limiting step of activation and oxidation of CH_4 to methanol (CH_3OH). These remarkable enzymes can dissociate the high-strength C-H bonds of CH_4 (dissociation energy of 439 kJ mol^{-1}, Blanksby and Ellison (2003)) at ambient temperature and pressure with high selectivity (100% conversion to CH_3OH). In contrast, physicochemical methods of converting methane to syngas or reagents for CH_3OH production (e.g. partial oxidation, dry- and steam reforming of CH_4) require very high energy input and occur at temperatures in the range of 700-900°C and pressures up to 100 bars and are often producing a number of by-products (Wilhelm, Simbeck *et al.* 2001; Dalton 2005; Hermans, Spier *et al.* 2009; Usman, Daud *et al.* 2015).

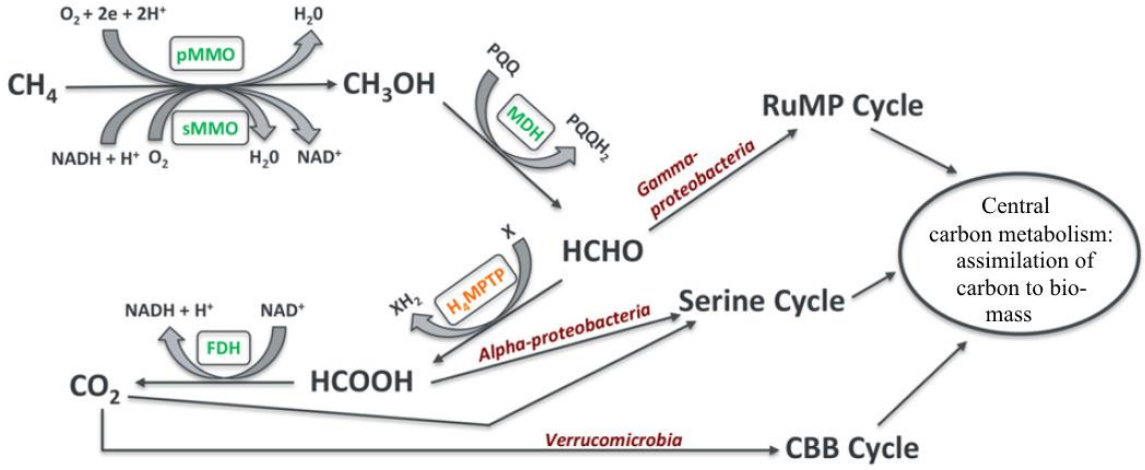


Figure 1-6. Simplified scheme of CH_4 oxidation by MOB (Fei, Guarnieri et al. 2014). *(p/s)MMO: (particulate/soluble) methane mono-oxygenase, MDH: methanol dehydrogenase. PQQ: pyrroloquinoline quinone, H_4MPTP: methylene tetrahydromethanopterine pathway (**Figure 1-5**), FDH: formate dehydrogenase.*

The primary oxidation of CH_4 can be performed because the MMO enzyme can split the O-O bond in O_2 and form two reducing equivalents (Yoon, Carey *et al.* 2009; Jiang, Chen *et al.* 2010), of which one is transferred to CH_4 to form CH_3OH while another is reduced to H_2O (Tinberg and Lippard 2011). The resulting CH_3OH is then oxidized (by a periplasmic methanol dehydrogenase) to formaldehyde (HCHO), a key molecule for both anabolism and catabolism in methylotrophs (Figure 1-6). Two distinctively different types of MMO are known, both catalyzing the same reaction. The most common type of MMO is the cell membrane-bound particulate MMO (pMMO) which is found in virtually all MOB except for the alphaproteobacterial MOB *Methylocella* and *Methyloferulla* (Semrau, DiSpirito *et al.* 2010; Vorobev, Baani *et al.* 2011). The genes encoding for pMMO are organized in the

pmoCAB operon which encodes the polypeptides for the α, β and γ subunits of the trimeric α3β3γ3 structure of pMMO in the genes the *pmoA*, *pmoB* and *pmoC* genes (Semrau, Chistoserdov *et al.* 1995; Culpepper and Rosenzweig 2012). Given the presence of pMMO in most MOB, *pmoA* is a common molecular marker for environmental detection (through (q)PCR and sequencing) of MOB, exhibiting high phylogenetic congruence with the 16S rRNA gene commonly used for taxonomic classification (Kolb, Knief *et al.* 2003; Dumont 2014; Knief 2015). A specialized copper uptake system mediated by methanobactin (Kim, Graham *et al.* 2004; DiSpirito, Semrau *et al.* 2016) has been associated with pMMO expression while without pMMO expression MopE/CorA "house-keeping" Cu uptake systems take over (Semrau, DiSpirito *et al.* 2010). Additionally, flavin secretion by alpha- and gammaproteobacterial MOB has been proposed as a distinct Cu and Fe siderophore mechanism (Balasubramanian, Levinson *et al.* 2010). The other type of MMO, the cytoplasmic soluble MMO (sMMO) is less widely expressed among the MOB and occurs in the genera *Methylosinus* and *Methylocystis* and a few strains of *Methylomonas*, *Methylomicrobium* and *Methylococcus* (Lidstrom 2006). The genes for sMMO are organized in the *mmoXYBZDC* operon (Stainthorpe, Lees *et al.* 1990). The *mmoX* gene is used as an additional genetic marker for MOB (Knief 2015) to incorporate the MOB of the *Beijerinckiaceae* family in the detection (Figure 1-3). The main trigger determining the predominant MMO in MOB which can express both sMMO and pMMO is the copper-to-biomass ratio (a phenomenon known as the "copper switch", Hakemian and Rosenzweig (2007)): the pMMO has a copper active site (Culpepper and Rosenzweig 2012) and is only expressed at high (>0.85-1 µmol g_{dw}^{-1}) Cu/biomass ratios (Balasubramanian and Rosenzweig 2008) whereas the sMMO contains a non-heme di-iron active site which is inhibited at high Cu/biomass ratios (Hakemian and Rosenzweig 2007). Hence, the copper-to-biomass ratio influences methanotrophic activity and community structure (Semrau, DiSpirito *et al.* 2010; van der Ha, Hoefman *et al.* 2010) which in turn may influence selection of specific non-MOB partners (van der Ha, Vanwonterghem *et al.* 2013). MOB expressing pMMO have a higher biomass yield and affinity for both CH_4 (K_m = 1-.2 µM) and O_2 (K_m = 0.1 µM) as opposed to MOB which rely on sMMO (Trotsenko and Murrell 2008). The reduced biomass yield of sMMO-relying MOB is a direct consequence of the lower redox potential of NAD(P)H used by sMMO to oxidize methane as compared to the reduced cytochrome C employed by pMMO (Dalton 2005). Although pMMO is the predominant CH_4 oxidation enzyme in nature (Fei, Guarnieri *et al.* 2014), sMMO has the distinct advantage of having a higher robustness

against inhibitors making its expression advantageous in polluted environments (Yu, Ramsay *et al.* 2009). The greater substrate promiscuity of the sMMO enzyme makes its expression an interesting strategy for biotechnological application of MOB in pollutant degradation and bioremediation (Benner, De Smet *et al.* 2015; Strong, Xie *et al.* 2015) .

From methanol to formaldehyde

After the initial oxidation of methane to methanol (CH_3OH, Figure 1-6), several mechanisms are available to the methylotrophic organisms to further metabolize methanol to formaldehyde (HCHO) such as the conventional methanol dehydrogenase (MDH) encoded by *MxaFI*, the and the less well studied alternatives Mdh2 (Kalyuzhnaya, Hristova *et al.* 2008) and the MxaF-homolog XoxF-type MDH. MDH are generally pyrroloquinoline quinone dependent (PQQ) dehydrogenases (Chistoserdova 2011). While MxaFI-type MDHs have Ca^{2+} at their active center, XoxF-type MDHs rely on rare earth elements (REE) from the lanthanide series (Keltjens, Pol *et al.* 2014), which may be responsible for the discrepancy between *xoxF* expression under laboratory conditions as opposed to natural systems (Wu, Wessels *et al.* 2015). The conventional MDH also require a cytochrome c electron acceptor encoded by *mxaG* (in the same gene cluster as mxaFI, Vuilleumier, Chistoserdova *et al.* (2009)). Among the methanotrophs, alpha- and gammaproteobacterial MOB (as well as NC10 MOB) were considered to rely on the conventional MDH, while verrucomicrobial MOB relied on XoxF (Chistoserdova 2011). However, XoxF-type MDH are encoded by many MOB (including NC10, Wu, Wessels *et al.* (2015)) and were recently shown to be the main MDH in the gammaproteobacterial MOB *Methylomicrobium buryatense* (Chu and Lidstrom 2016). XoxF-type MDH are believed to have a greater catalytic efficiency than MxaF-type MDH. In the environment lanthanides are typically available in sufficient amounts to favor the XoxF-type over the MxaF-type MDHs (in a regulatory process coined the "lanthanide MDH switch"), even in the presence of excess calcium (Keltjens, Pol *et al.* 2014; Chu and Lidstrom 2016). A link with the copper-dependent switching between pMMO and sMMO was suggested for an alphaproteobacterial MOB (*M. trichosporium* OB3b), where MxaF-type MDH dominance was found to be linked with pMMO expression while XoxF-type MDH could only become the dominant MDH under conditions favoring sMMO expression (Ul Haque, Kalidass *et al.* 2015), however this does not appear to be the case for the gammaproteobacterial MOB (Chu and Lidstrom 2016). Overall, XoxF-type MDHs exhibit superior methanol dehydrogenase activity (due to the REE in their active centers, Keltjens, Pol *et al.* (2014)) and addition of lanthanides to growth media may affect methanotrophic community composition, which has not been studied thus far. Besides CH_4, proteobacterial MOB were reported to be able to use

CH_3OH as a sole carbon and energy source (Hanson and Hanson 1996), however large differences in CH_3OH sensitivity (due to formaldehyde) were observed among strains (Bowman 2006).

Carbon assimilation

From the level of formaldehyde, three strategies of carbon assimilation are available to the MOB (Figure 1-5), which appear to be associated with their phylogenetic background (Chistoserdova 2011). Additionally, formaldehyde can also be oxidized catabolically through formate (HCOOH via a tetrahydromethanopterin-pathway, Figure 1-6) and then to CO_2 (via formate dehydrogenase), in order to generate reducing equivalents for CH_4 metabolism (Anthony 1991; Hanson and Hanson 1996). Gammaproteobacterial MOB assimilate carbon directly from formaldehyde into biomass via the ribulose monophosphate pathway (RuMP, Figure 1-7), where a C3 intermediate is formed out of 3 mol HCHO (Trotsenko and Murrell 2008): In the RuMP pathway formaldehyde is added onto ribulose monophosphate (a C5 sugar) to generate glucose-6-phosophate. The C6 sugars are then transformed to glyceraldehyde-3-phosphate (C3) molecule which can be used for assimilation, oxidized for NAD(P)H generation or regenerated into RuMP (Figure 1-7). Hexulose-6-phosphate synthase (H6PS) and hexulose-6-phosphate isomerase (H6PI) are believed to be unique to the RuMP pathway (Chistoserdova 2011; Fei, Guarnieri *et al.* 2014) while other enzymes of the glycolysis, pentose-phosphate cycle and Entner-Doudoroff pathways are used to complete the RuMP pathway. Gammaproteobacterial MOB that use the RuMP pathway do not have a functional TCA cycle (Jiang, Chen *et al.* 2010). Alphaproteobacterial MOB employ the serine cycle of for carbon assimilation and have functioning TCA cycles (Matsen, Yang *et al.* 2013). In the serine cycle, methylene-H_4F and CO_2 are assimilated into biomass through a series of reactions with amino acids and organic acids resulting in C3 and C4 building blocks and glyoxylate that serves as acceptor for methylene-H_4F. The net result is the assimilation of 2 moles of HCHO and 1 mole of CO_2 into 1 mole of 2-phosphoglycerate (Anthony 1982; Chistoserdova 2011). Key enzymes that are involved with the serine cycle operation are hydroxypyruvate reductase (HPR) and serine glyoxylate aminotransferase. Gammaproteobacterial genomes may also harbor serine cycle genes though most of them are scattered over the genome and a key gene encoding phosphoenolpyruvate carboxylase (PPC) is missing (Ward, Larsen *et al.* 2004; Chistoserdova 2011). MOB from the phyla Verrucomicrobia (Khadem, Pol *et al.* 2011) and NC10 (Rasigraf, Kool *et al.* 2014) use the Calvin-Benson-Bassham (CBB) pathway to assimilate CH_4 – derived carbon at the level of CO_2 and are therefore autotrophic MOB.

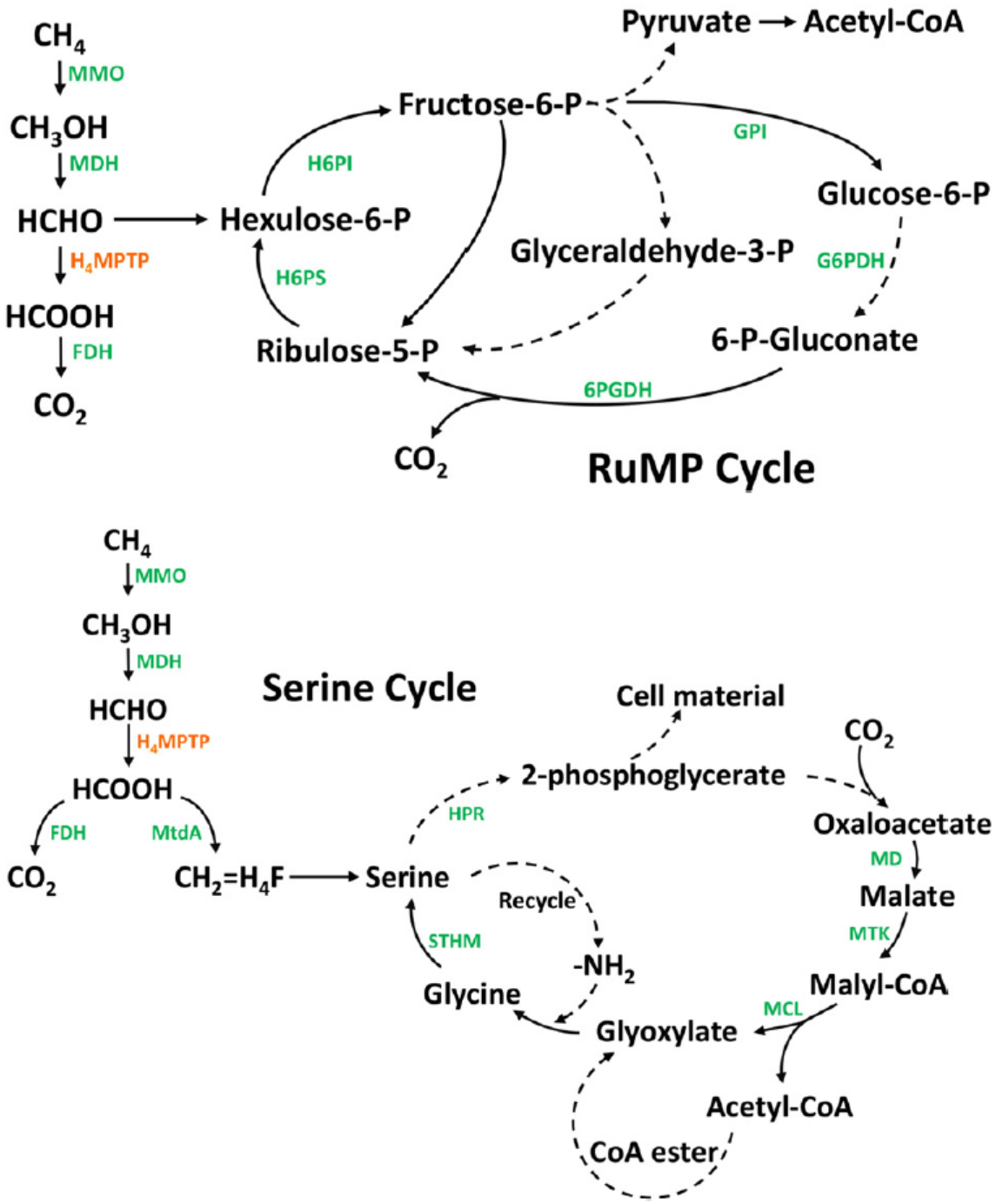


Figure 1-7. Simplified molecular pathways for the RuMP and serine cycles in MOB (Fei, Guarnieri et al. 2014).*Major enzymes have a green font color. Dashed arrows represent possible exit points of metabolites for biosynthetic reactions. For clarification of abbreviations we refer the reader to the main text and the original publication.*

As opposed to the serine cycle, higher growth yields can be achieved by MOB employing the RuMP pathway: the formation of C3 building blocks for biomass synthesis requires only 1 mole ATP per mole of C3 intermediate, whereas the serine pathway consumes 3 mole ATP per C3 intermediate (Figure 1-7, Equation 1-1). However, the overall stoichiometry of oxygen

and nitrogen demands are not strongly different among both pathways (Equation 1-2). MOB are able to partially regulate the distribution of HCHO among anabolism and catabolism, which in turn influences O_2 consumption: while the complete oxidation of CH_4 to CO_2 requires 2 mol O_2 per mol of CH_4 oxidized, assimilation into biomass via formaldehyde only requires 1 mol of O_2 (Scheutz, Kjeldsen *et al.* 2009).

Equation 1-1. Global balances of serine and RuMP formaldehyde assimilation (Hanson and Hanson 1996)

$$Serine: 2HCHO + CO_2 + 3ATP + 2\,NADH \rightarrow 2 - phosphoglycerate + 2ADP + P_i + NAD^+$$
$$RuMP: 3HCHO + ATP \rightarrow glyceraldehyde - 3 - phosphate + ADP$$

Equation 1-2. Overall stoichiometry from CH_4 to biomass($C_4H_8O_2N$) with respect to O_2 and NH_4^+ for the serine and RuMP cycles (Trotsenko and Murrell 2008; Jiang, Chen et al. 2010)

$$Serine: CH_4 + 1.50\,O_2 + 0.12\,NH_4^+ \rightarrow 0.12(C_4H_8O_2N) + 0.53\,CO_2 + 1.71\,H_2O + 0.12H^+$$
$$RuMP: CH_4 + 1.57\,O_2 + 0.10\,NH_4^+ \rightarrow 0.10(C_4H_8O_2N) + 0.59\,CO_2 + 1.74\,H_2O + 0.10H^+$$

2.2.2. Anaerobic methane oxidation

In the absence of oxygen methane oxidation can still proceed by means of alternative electron acceptors (Joye 2012). This has been demonstrated for marine environments where the quantitatively most important sink is the coupling of CH_4 oxidation to the reduction of sulphate (AOM) (Orphan, House *et al.* 2001; Knittel and Boetius 2009) in a dual species microbial consortium of syntrophic anaerobic methane oxidizing Archaea (ANME) and bacterial sulphate reducing partners of the *Desulfosarcina/Desulfococcus* clade or from the HotSeep-1 group. Currently three major clades of ANME Archaea are distinguished: ANME-1, ANME-2 and ANME-3 (Garrett and Klenk 2008). While initially this association was thought to be syntrophic, exchanging reducing equivalents through chemicals like H_2 or formate, recent evidence showed that possibly a mechanism of direct interspecies electron transfer (DIET) may occur (McGlynn, Chadwick *et al.* 2015). This finding was confirmed by the fact that AOM partners could be decoupled in the laboratory by the addition of the artificial electron acceptor 9,10-anthraquinone-2,6-disulfonate (AQDS) (Scheller, Yu *et al.* 2016) and humic analogs as well as soluble ferric iron (Fe^{3+}) complexes were found to stimulate AOM without sulphate. These findings could be extrapolated to a mechanism of extracellular electron transfer to insoluble iron oxides through cytochrome c proteins and pili (Rotaru and Thamdrup 2016). Apart from sulphate, AOM with nitrate (Haroon, Hu *et al.* 2013) and metal oxides (Beal, House *et al.* 2009) as terminal electron acceptors has been

reported. The AOM bacterial-archaeal communities are of great importance in global CH_4 cycling as they are believed to attenuate about 90% of oceanic CH_4 before it reaches the surface (Knittel and Boetius 2009).

2.2.3. Facultative and high-affinity MOB

While many MOB have multiple copies of the *pmo* operon, they are not always exact copies of one another. For instance in *Methylocystis* sp. SC2 a copy of *pmoA* with only 73% identity to *pmoA* of *Methylocystaceae* was found (*pmoA2*, part of the *pmoCAB2* operon) which was found to be part of a pMMO able to oxidize CH_4 at lower concentrations (1-100 ppmv) compared to the conventional pMMO (>600 ppmv) (Dunfield, Yimga *et al.* 2002; Baani and Liesack 2008) and may be essential to CH_4 oxidation under atmospheric concentrations (Kravchenko, Kizilova *et al.* 2010). Apart from *pmoA2*, another homolog of *pmoA* has been detected among many gammaproteobacterial MOB, named *pxmA* (Tavormina, Orphan *et al.* 2011), however the function of its gene product remains largely unknown although it was overexpressed during hypoxia in a denitrifying methanotroph (Kits, Klotz *et al.* 2015). Although upland soils are net sinks of CH_4 due to aerobic microbial CH_4 oxidation, only very few *high affinity* MOB isolates (able to thrive under the atmospheric CH_4 mixing ratio of 1.8 ppmv) are obtained to date, all belonging to the *Methylocystis* genus (Conrad 2009; Kolb 2009).

Apart from the expression of a pMMO variant which allows growth under atmospheric levels of CH_4 another survival strategy of MOB could rely on facultative metabolism of alternative carbon and energy sources than CH_4 (or other C1 compounds) allowing growth and survival in environments with periodic CH_4 limitation (Dedysh, Knief *et al.* 2005; Dunfield, Belova *et al.* 2010; Belova, Baani *et al.* 2011; Im, Lee *et al.* 2011; Semrau, DiSpirito *et al.* 2011). Members of the genus *Methylocella* could utilize (besides C1 compounds) organic acids such as acetate, pyruvate, propionate, succinate, malate and gluconate, alcohols such as ethanol, and 2-propanol and gaseous ethane and propane (Crombie and Murrell 2014). Additionally, some strains of *Methylocapsa* and *Methylocystis* were found to utilize acetate next to CH_4 (Dunfield, Belova *et al.* 2010; Belova, Baani *et al.* 2011; Im, Lee *et al.* 2011; Vorobev, Jagadevan *et al.* 2014). While some *Methylocella* strains showed preference for growth with multicarbon compounds over CH_4, acetate-utilizing *Methylocystis* clearly preferred CH_4. Hence, facultative acetate metabolism by MOB could be a survival strategy (Belova, Baani *et al.* 2011). Given their versatility in substrate utilization, these strains have been thoroughly

researched for their biotechnological potential (e.g. Im and Semrau (2011)). Interestingly, facultative MOB (*Methylocystis*, *Methylocapsa* and *Methylocella*) all fall into the Alphaproteobacteria, illustrating their broader substrate versatility than gammaproteobacterial MOB which reflects in their conceptual life strategy as stress tolerators (Ho, Kerckhof *et al.* 2013).

2.2.4. Methane fermentation under hypoxia

A few years ago Kalyuzhnaya and colleagues reported on the coupling of methane assimilation with a highly efficient pyrophosphate-mediated glycolytic pathway under oxygen-limited conditions, resulting in a novel form of fermentation rather than respiration-based methanotrophy (Kalyuzhnaya, Yang *et al.* 2013). In the gammaproteobacterial *Methylomicrobium alcaliphilum* this fermentation-based CH_4 utilisation mode led to the formation of formate, acetate, lactate, succinate and hydroxybutyrate, which were partly excreted and hence accumulated in the growth medium. The presence of the putative fermentation genes in gammaproteobacterial MOB suggests this type of metabolism is likely widespread (Khmelenina, Rozova *et al.* 2015). The accumulation of these compounds under oxygen limitation could greatly impact the composition of methanotrophic communities consisting out of MOB and non-MOB (section 3.2) as the latter will be differentially impacted by the exuded organic carbon sources. This could be linked to observations in microcosms of lake Washington at different oxygen tensions, which showed that oxygen availability is a major factor determining the composition of microbial communities involved in methane oxidation (Hernandez, Beck *et al.* 2015; Oshkin, Beck *et al.* 2015), although differences in nitrogen metabolism rather than carbon metabolism were speculated to be determining for community composition under hypoxia (Hernandez, Beck *et al.* 2015).

2.3. Ecology and ecophysiology

All ecosystems harboring methane sources are typical habitats of MOB. These include (but are not limited to) sediments and water columns of freshwater and marine systems, aquifers, floodplains, high arctic and tundra wetlands, peat bogs, upland soils, rice paddies, landfill covers and sewage sludge (Conrad 2007; Semrau, DiSpirito *et al.* 2010; Ho, Vlaeminck *et al.* 2013; Bowman 2014; Knief 2015). Aerobic MOB will typically occur at the oxic/anoxic interface. MOB can occur over a wide range of physicochemical niches ranging from circumneutral pH to (extremely) acidophilic growth restricted verrucomicrobial MOB (down

to pH 1, with an optimum around pH 2-2.5; Dunfield, Yuryev *et al.* (2007)). Although gammaproteobacterial MOB (*Methylomonas* sp.) have been found to tolerate pH 3.8 (Danilova, Kulichevskaya *et al.* 2013), generally adaptation to mildly acidic pH is believed to be a characteristic of alphaproteobacterial MOB belonging to *Beijerinckiaceae* and some *Methylocystis* strains (Knief 2015). Thermoacidophilic verrucomicrobial MOB appear to be largely restricted to geothermal environments, though in particular to acidic conditions, since they seem to have a broader temperature range as revealed by cultivation-dependent and –independent analyses (Sharp, Martinez-Lorenzo *et al.* 2014; van Teeseling, Pol *et al.* 2014). On the other side of the pH spectrum alkaliphilic MOB have been found to thrive in soda lakes at pH 9 or higher (Lin, Radajewski *et al.* 2004; Sorokin and Kuenen 2005; Antony, Kumaresan *et al.* 2010) and extremophilic haloalkaliphilic MOB display the ability to grow even at pH 11 (Trotsenko and Khmelenina 2002). Gammaproteobacterial MOB were found to dominate soda lakes, though a great diversity of gamma- and alphaproteobacterial MOB can be observed in these environments (Bowman 2014). Likewise optimal temperatures for MOB cover a wide range. While most described MOB are mesophilic (Hanson and Hanson 1996), several psychrophilic MOB (growing at temperatures lower than 15°C) have been described to grow at temperatures as low as 3.5 to 10°C (Semrau, DiSpirito *et al.* 2010) whereas thermophilic and thermotolerant MOB are known with optimal growth ranges from 55°C up to 62°C which may localize in hot springs and organic-rich environments with metabolic heating (e.g. compost) (Trotsenko, Medvedkova *et al.* 2009). In hot springs with large MOB populations, mostly only gammaproteobacterial MOB were detected (Bowman 2014). Depending on several physicochemical conditions, such as CH_4 and O_2 availability as well as copper-to-biomass ratio, selection of different groups of MOB may occur, shaping the MOB community structure (Henckel, Roslev *et al.* 2000; Semrau, DiSpirito *et al.* 2010; Krause, Lüke *et al.* 2012; Hernandez, Beck *et al.* 2015). Community level analysis of MOB populations and the ecological characteristics of gamma- and alphaproteobacterial MOB suggested that these groups have different traits which could be conceptualized as life strategies (Ho, Kerckhof *et al.* 2013). Gammaproteobacterial MOB were found to be highly responsive to substrate availability, whereas their numbers quickly reduced when environmental stress or substrate limiting conditions are applied. Alphaproteobacterial MOB were generally more stable and are assumed to be in a dormant state in the soil (Krause, Lüke *et al.* 2012). They display better stress tolerance compared to gammaproteobacterial MOB and facultative MOB (section 2.2.3) were limited to this phylogenetic group. Hence, in the plant ecology framework of Competitor - Stress tolerator - Ruderal (C-S-R, Grime (1977))

alphaproteobacterial MOB can be considered stress tolerators (S), stress tolerator-ruderals (S-R) or stress tolerators-competitors (S-C) while gammaproteobacterial MOB can be classified as competitors (C) or competitors-ruderals (C-R).

3. The methanotrophic *interactome*

Much like methanogenesis requires cooperation among (syntrophic) partner organisms (section 1.2), recent evidence points that also at the other side of the methane cycle methanotrophy greatly benefits from microbial interactions (Oshkin, Beck *et al.* 2015). Interactions in the metanotrophic *interactome* are less "compelling" as the main functionality is carried out by methanotrophic bacteria, which can also be cultivated as pure strains and still carry out the full functionality of methane oxidation (as opposed to methanogenic Archaea, which rely on other organisms to supply them hydrogen and CO_2 /acetate from organic matter to synthesize CH_4). Given the fact that many methanotrophs can grow in an axenic way, it is all the more interesting that in natural environments communities the carbon and energy which the MOB derive from CH_4 is divided throughout the whole community.

3.1. The methanotrophic food web

MOB are known to engage in various naturally occurring symbioses and interactions with other organisms, comprising other bacteria (Hanson and Hanson 1996; Hrsak and Begonja 2000; Modin, Fukushi *et al.* 2007; Stock, Hoefman *et al.* 2013; van der Ha, Vanwonterghem *et al.* 2013; Iguchi, Yurimoto *et al.* 2015), marine invertebrates (snails, mussels, sponges, tubeworms, crabs) at hydrothermal vents and cold seeps in the deep sea (Petersen and Dubilier 2009; Ruff, Arnds *et al.* 2013; Watsuji, Yamamoto *et al.* 2014), *Sphagnum* in peatlands and brown mosses in polygonal tundra (Raghoebarsing, Smolders *et al.* 2005; Kip, van Winden *et al.* 2010; Liebner, Zeyer *et al.* 2011; Larmola, Leppanen *et al.* 2014; Putkinen, Larmola *et al.* 2014; Ho and Bodelier 2015), (micro)algae (Hutchens, Radajewski *et al.* 2004; van der Ha, Bundervoet *et al.* 2011; van der Ha, Nachtergaele *et al.* 2012; Milucka, Kirf *et al.* 2015) and wetland plants (Bao, Okubo *et al.* 2014; Minamisawa, Imaizumi-Anraku *et al.* 2016). In ecosystems lacking other organic carbon sources, MOB can act as primary producers and provide the base for an extended food web by supplying CH_4 – derived carbon to their environment (Murase and Frenzel 2007; Semrau, DiSpirito *et al.* 2010; Ruff, Arnds *et al.* 2013; van Duinen, Vermonden *et al.* 2013; Agasild, Zingel *et al.* 2014) and methane carbon has been found to support aquatic food webs up to the fish level (Sanseverino,

Bastviken *et al.* 2012). Several underlying mechanisms for trophic interactions between MOB and other organisms exist. Predation (grazing) of MOB by algae (e.g. *Ochromonas danica*), bacteriovores (e.g. *Bdellovibrio bacteriovorus*) and protozoa are negative interactions with other organisms which may shape methanotroph community structure by selective grazing (Hutchens, Radajewski *et al.* 2004; Murase and Frenzel 2008). However mutualistic and symbiotic interactions can be observed by the endosymbiontic lifestyle of (mainly gammaproteobacterial) MOB in marine invertebrates: the host provides MOB with a stable environment and optimal position for CH_4 oxidation in the mixing zone where CH_4 meets the aerated layer and in return the MOB supply the host with CH_4-derived carbon either through metabolites or bacterial biomass (Dubilier, Bergin *et al.* 2008; Ruff, Arnds *et al.* 2013). Apart from endosymbiontic associations with marine invertebrates, recent evidence from epibiontic associations of MOB with the setae of the deep-sea dwelling crab *Shinkaia crosniere* suggested that also epibionts can nutritionally support their host (Watsuji, Yamamoto *et al.* 2014). Similarly, alphaproteobacterial MOB were found to inhabit epidermal cell walls and xylem of rice roots, where they fix N_2 and CH_4 –derived carbon and are provided with a methane-rich environment (Bao, Okubo *et al.* 2014; Minamisawa, Imaizumi-Anraku *et al.* 2016). An association with photosynthetic algae enabled MOB to colonize the water column of a permanently stratified anoxic lake, Lago di Cadagno (Milucka, Kirf *et al.* 2015), where the oxygenic photosynthesis by the algae in the light-penetrated zone of the lake created a niche for aerobic MOB in the otherwise anoxic ecosystem. Oxygen diffusion through the aerenchymatous tissue of rice plants in flooded rice fields can create a partially oxic environment for aerobic MOB and their associated microbial communities to thrive (Qiu, Conrad *et al.* 2009). MOB may also benefit from interactions with other micro-organisms, which are outlined in the next section.

3.2. Preferential partnerships in methanotrophic microbial ecosystems

MOB are often found to be associated with non-MOB bacterial partners. MOB are believed to support growth of methylotrophic organisms by the release of C1 compounds such as methanol and formaldehyde (Wilkinson, Topiwala *et al.* 1974; Megraw and Knowles 1989; Hanson and Hanson 1996; Hesselsoe, Boysen *et al.* 2005), which in turn is beneficial to the MOB since excessive accumulation of CH_3OH and (mainly) HCHO can be toxic to the cells. Additionally, evidence has emerged that possibly, other low-molecular-weight carbon compounds may be released by the MOB supporting non-obligately/restricted-facultative

methylotrophic partners: acetate, succinate, hydroxybutyrate, lactate, citrate and sucrose (Rhee and Fuhs 1978; Costa, Dijkema *et al.* 2000; Trotsenko and Khmelenina 2002; Medvedkova, Khmelenina *et al.* 2007; Kalyuzhnaya, Yang *et al.* 2013). Additionally, several (other) secondary metabolites such as ectoine and methanobactin can be produced and excreted by the MOB (Khmelenina, Rozova *et al.* 2015), though their role in interactions with non-MOB has not been explored. Finally, some MOB are known to produce extracellular polymeric substances (EPS), largely consisting out of polysaccharides, probably as a response to excess carbon to other nutrients ratios or oxygen stress (Huq, Ralph *et al.* 1978; Wilshusen, Hettiaratchi *et al.* 2004; Wei, Su *et al.* 2015). As opposed to the CH_4-derived carbon "deliberately" excreted by the MOB, growth-dependent lysis could release proteins and nucleic acids into the environment, enabling necrotrophic growth of the non-MOB partners (Linton and Buckee 1977; Eisentraeger, Klag *et al.* 2001). Necrotrophic growth could be considered an aspecific process, as many bacteria could benefit from growth-dependent lysis products of the MOB. However, clear and specific associations between MOB and non-MOB have been observed (Iguchi, Yurimoto *et al.* 2011; Stock, Hoefman *et al.* 2013; Jeong, Cho *et al.* 2014). These selective and preferential partnerships could be attributed to benefits for the MOB by associating with non-MOB which provide stimulatory factors for MOB growth. For example, in an enrichment from forest soil (Iguchi, Yurimoto *et al.* 2011) non-MOB partners of the genus *Rhizobium* increased growth of the MOB *Methylovulum miyakonense* by means of cobalamin (vitamin B12). Furthermore, a density-dependent growth stimulation of *Sphingopyxis* NM1 on *Methylocystis* M6 was observed at a 1:9 (v/v) MOB:non-MOB mixing ratio where mRNA transcripts of enzymes involved in CH_4 metabolism were upregulated (Jeong, Cho *et al.* 2014). An unfortunate side effect of these specific interactions is the problematic purification of some MOB, as non-MOB partners (often methylotrophs) such as e.g. methanol-utilizing hyphomicrobia often co-purify with the MOB and impede MOB purification (Quayle 1972; Bowman 2006; Geymonat, Ferrando *et al.* 2011; Dedysh, Kulichevskaya *et al.* 2012; Takeuchi, Kamagata *et al.* 2014; Danilova, Suzina *et al.* 2016). Additionally, the intense association between MOB and non-MOB results in cross-feeding of $^{13}CH_4$-derived carbon, which occurs during prolonged incubations required for (DNA) stable isotope probing (SIP) of methanotrophic enrichments and natural samples (Neufeld, Vohra *et al.* 2007; Neufeld, Wagner *et al.* 2007). Rather than considering this a limitation of SIP, in this thesis this cross-feeding was considered an advantage and used as a measure for a positive interaction between non-MOB and MOB. Additionally, we added evidence from co-cultivation experiments of MOB and non-MOB and established a dataset 303 described

partnerships (supplied as a supplementary dataset to the publication of Chapter 2) between MOB and non-MOB. The most frequently described partners are known non-methanotrophic methylotrophs *Methylophilaceae* (13%, Figure 1-8) and *Hyphomicrobiaceae* (8%, Figure 1-8). However, other families were also frequently identified as members of the methanotrophic *interactome*, for example *Flavobacteriaceae* (4%, mainly *Flavobacterium*), *Xanthomonadaceae* (4%) and *Comammonadaceae* (5%).

The relationship between gammaproteobacterial MOB belonging to the family *Methylococcaceae* and the betaproteobacterial methylotrophic family *Methylophilaceae* has been repeatedly demonstrated (Hutchens, Radajewski *et al.* 2004; Beck, McTaggart *et al.* 2014; Hernandez, Beck *et al.* 2015). Furthermore He, Wooller *et al.* (2012) showed a linear relationship between Methylophilaceae and Methylococcaceae in arctic lake sediment. However, overall the mode and determinants of successful MOB:non-MOB interaction as well as the mechanisms underlying MOB partner selectivity are still poorly understood and require further attention.

Definition: the methanotrophic "partnership"

Although a partnership could imply a bi-directional interaction, within the scope of this thesis a methanotrophic "partner" in the methanotrophic *interactome* is defined as any bacterium *intricately involved* in the biological methane oxidation. This *intricate involvement* is substantiated by either the ***direct assimilation of $^{13}CH_4$ derived carbon*** into the biomass and biomarkers (e.g. DNA-SIP, Chapter 2) or ***positive interaction in co-cultivation experiments*** (positive as compared to the axenic MOB cultivation in the sense of increased total biomass, MOB biomass, non-MOB partner biomass, ….). In Chapters 3 and 4 however any bacterial strain that was added to the co-cultivation was referred to as a methanotrophic partner for writing convenience, however only the persistent organisms should be considered as fitting to the current definition.

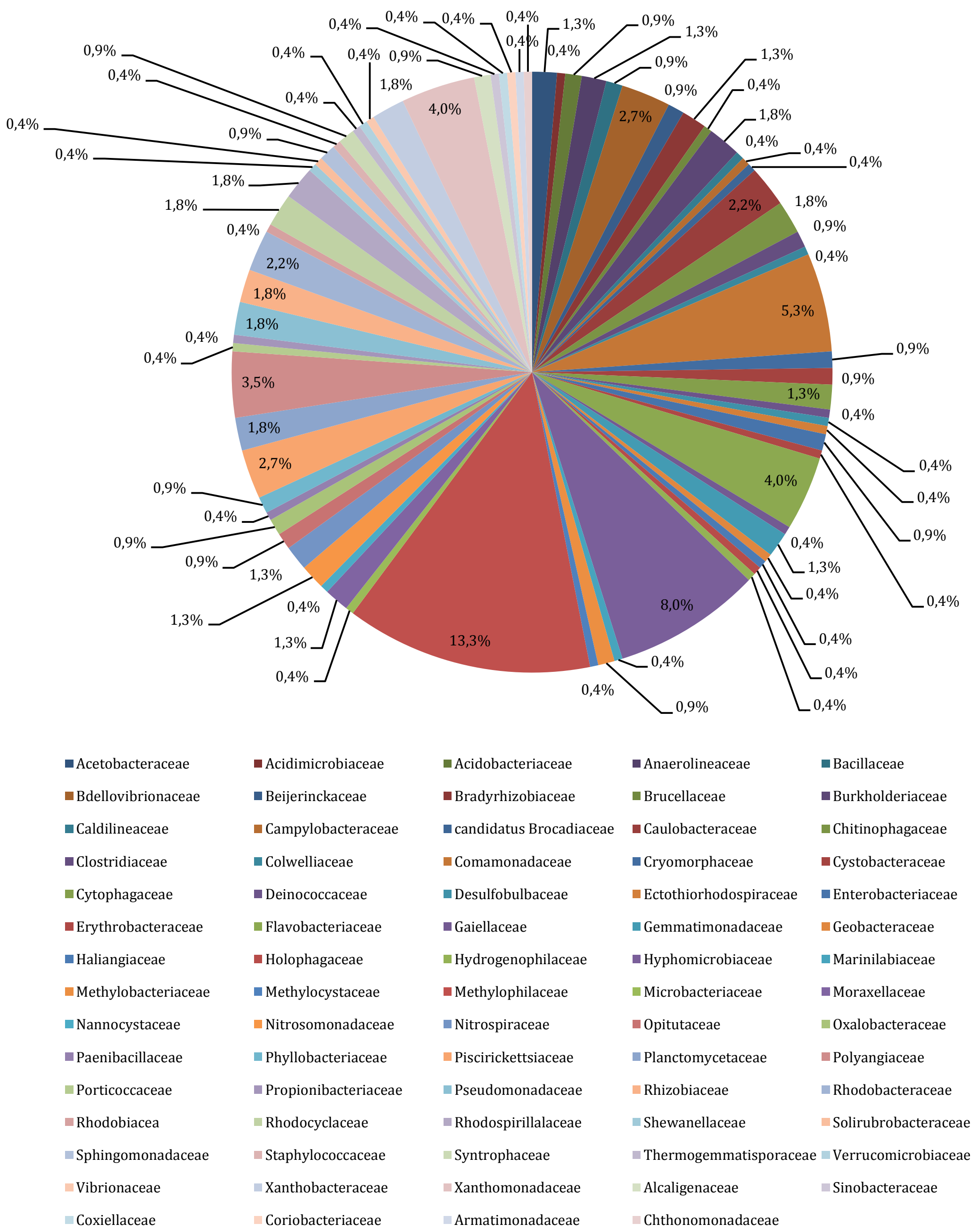


Figure 1-8. Count summary of 303 described partnerships of non-MOB with MOB at the family level.

4. Applications of methanotrophic *interactomes*

From a biotechnological point of view waste streams rich in CH_4 can be valorized for energy and heat production, however at lower CH_4 concentrations microbial mitigation strategies should be considered. MOB-driven CH_4 mitigation strategies have been implemented in several end-of-pipe solutions (Jiang, Chen *et al.* 2010; Pratt, Walcroft *et al.* 2012; Duan, Al-

Soud *et al.* 2014), and some strategies even succeed in recovery of the energy and carbon stored in CH_4 (Helm, Wendlandt *et al.* 2006; Hamer 2010; Fei, Guarnieri *et al.* 2014).

While axenic MOB cultures are known to have many potential biotechnological applications (such as *in*- and *ex-situ* bioremediation of organic compounds (TCE, VC, *t*-DCE), production of biopolymers (polyhydroxy-alkanoates such as PHB, although mixed or enriched methanotrophic *interactomes* are generally employed to increase performance of PHB production (*vide infra*), production of extracellular polysaccharides, production of secondary metabolites (ectoine), production of components for biodiesel (gas-to-liquid, GTL), production of EPS, sucrose and lipids (Jiang, Chen *et al.* 2010; Fei, Guarnieri *et al.* 2014; Khmelenina, Rozova *et al.* 2015; Strong, Xie *et al.* 2015)), exploitation and engineering of methanotrophic *interactomes* could lead to improved and sustainable mitigation and recovery of CH_4 in the form of metabolic energy or CH_4-derived carbon. Unlocking the full potential of these *interactomes* for biotechnological applications may be hampered by the difficulty in methanotrophic *interactome* design and control (Jiang, Chen *et al.* 2010). Nevertheless, methanotrophic *interactomes* could have distinct advantages over axenic MOB. For example, Bothe and colleagues (Bothe, Jensen *et al.* 2002) suggested that contaminant partners (*Anaeribacillus* sp., *Brevibacillus agri*, *Ralstonia* sp.) are actually beneficial to an industrial biotechnological process such as the production of single-cell protein (SCP), by removing inhibitory compounds from the medium released by *Methylococcus capsulatus*. The production of biological protein from methane (by means of e.g. *Methylococcus capsulatus*) has become an attractive alternative to fish feed (Calysta^{TM} FeedKind^{TM}). Partnerships between MOB and micro-algae ("methalgae") may be even more interesting sources of feed, as they do not depend on external oxygen supply and may be directly used to convert highly CH_4-oversaturated industrial anaerobic waste-waters to microbial lipids and protein (van der Ha, Bundervoet *et al.* 2011). The methalgae consortium also tends to form flocs, which facilitates biomass separation from the liquid phase. Direct production of methalgae-flocs enriched in PHB (which is beneficial to aquaculture (De Schryver, Sinha *et al.* 2010; Dinh, Wille *et al.* 2010)) and algal lipids from synthetic biogas induced by nutrient limitation and pH shifts in a *Scenedesmus*/*Methylocystis interactome* has been described (van der Ha, Nachtergaele *et al.* 2012). Furthermore, methane-driven denitrification (termed MOD Liu, Sun *et al.* (2014)) or AME-D (in the case of aerobic MOD, Zhu, Wang *et al.* (2016)) is a process believed to be driven by an *interactome* between MOB which supply organic carbon to denitrifiers (Costa, Dijkema *et al.* 2000; Modin, Fukushi *et al.* 2007). However, the discovery of the nitrite-dependent *Methylomirabilis oxyfera* in the NC10 phylum (Ettwig, van

Alen *et al.* 2009) as well as anaerobic methane-oxidizing Archaea (Haroon, Hu *et al.* 2013) and aerobic MOB (such as *Methylomonas denitrificans* FJG1, Kits, Klotz *et al.* (2015) which are able to perform (partial) denitrification with methane as a carbon source without non-MOB denitrifying partners may change the assumed importance of microbial interactions to this process (Figure 1-9).

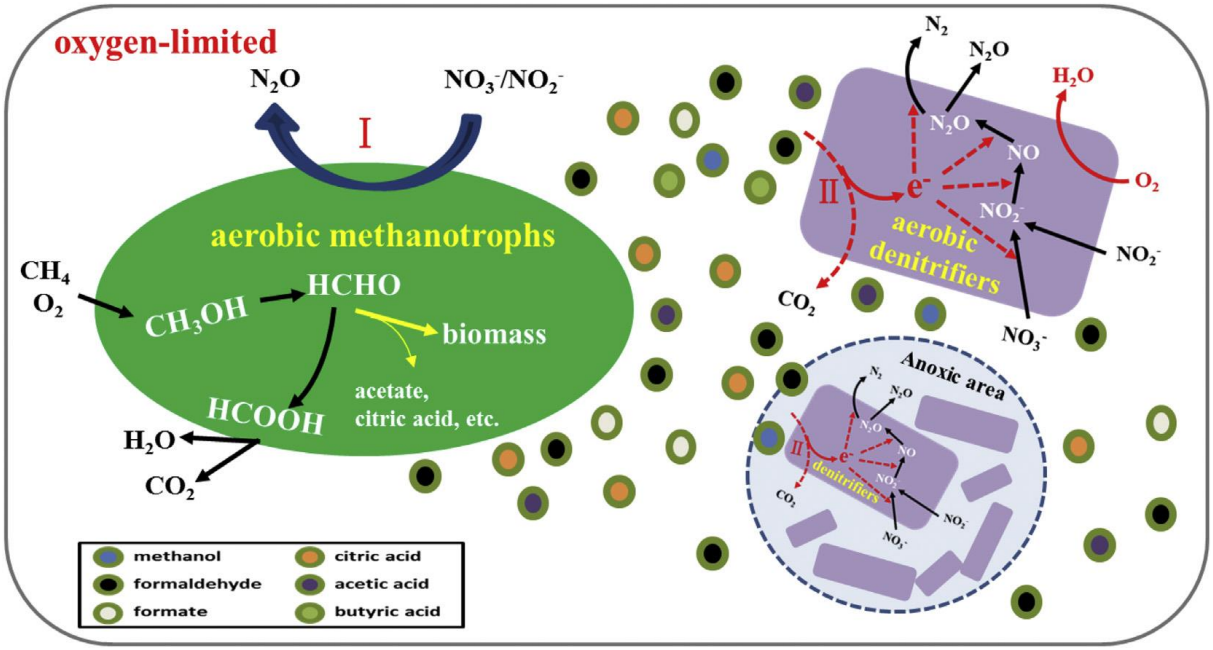


Figure 1-9. Postulated pathways of aerobic methane oxidation coupled to denitrification and trophic links between these two processes (Zhu, Wang et al. 2016). *Main microorganisms responsible for each pathway: (I) Methylomicrobium album ATCC 33003 (Nyerges, Han et al. 2010), Methylomonas denitrificans FJG1 (Kits, Klotz et al. 2015), (II)Methylotenera mobilis (Bosch, Wang et al. 2009), Methylocystis parvus and Mesorhizobium plurifarium (Costa, Dijkema et al. 2000).*

Methanotrophic consortia also occur naturally in wastewater treatment plants where they attenuate methane emissions (Ho, Vlaeminck *et al.* 2013). Furthermore, associations between *Bradyrhizobia* and *Alphaproteobacterial* MOB may be key to N fixation in the rice rhizosphere (Minamisawa, Imaizumi-Anraku *et al.* 2016). In the case of MOB-mediated biodegradation of organic pollutants Hrsak and Begonja (2000) reported that non-MOB stimulated their MOB partners by removing toxic intermediates. A simple community, mainly consisting of a MOB and seven accompanying non-MOB bacteria, was used for PHB production from CH_4. This community proved to be stable in composition for a prolonged period of 29 months even under non-aseptic conditions, suggesting a strong self-regulating capacity and a positive selection towards an optimal *interactome* for PHB production (Helm, Wendlandt *et al.* 2006).

Apart from advances on the microbiological side of the biotechnological applications of MOB (Duan, Luo *et al.* 2011; Minshull, Ness *et al.* 2014), enhancement of operational parameters counteracting the low solubility of CH_4 and O_2 in the aqueous phase could greatly improve biotechnological applications of MOB and methanotrophic *interactomes*. Such enhancements can rely on changes in reactor design such as unconstrained membrane biofilm reactors (Hamer 2010), mixing of the multiphase system in a U-loop reactor (Prado-Rubio, Jørgensen *et al.* 2010) and gas recycling (Estrada, Lebrero *et al.* 2014). Additionally increased mass transfer may be obtained using paraffin oil methane vectors (Han, Su *et al.* 2009).

5. Research outline and objectives

This dissertation focusses on the bacterial interactions between MOB and non-MOB and tries to assess what are the determining factors for non-MOB partner selection in the methanotrophic *interactome*. Specific focus lies on representatives of alpha- and gammaproteobacterial MOB (which have differing physiologies, section 2.2.1), resulting in differential functional traits which can in turn be conceptualized as distinct life strategies (Ho, Kerckhof *et al.* 2013). The impact of these differences on interactions with non-MOB has not been studied. An increased insight in the co-existence and the extent of co-dependence of MOB with non-MOB is required to fully understand the essential ecosystem service of biological methane oxidation (Oshkin, Beck *et al.* 2015).

PART 1: TOP-DOWN APPROACH IN UNRAVELLING THE METHANOTROPHIC *INTERACTOME*

First, an established methanotrophic enrichment lab culture which was enriched over an extended period of time under non-axenic conditions was employed as the inoculum. In this way, the community composition should reflect an optimal methanotrophic *interactome*. In Chapter 2 this *interactome* was dissected by means of time-resolved stable isotope probing (SIP) to assess which *interactome* partners are most intricately involved in the metabolism of CH_4-derived carbon.

PART 2: BOTTOM-UP APPROACHES IN PUZZLING THE METHANOTROPHIC *INTERACTOME* TOGETHER

Next, an attempt to assemble an optimal methanotrophic *interactome* was executed by piecing together multiple non-MOB strains with MOB. In a first approach, described in Chapter 3, partner selection was "supervised": first, initial compatibility was assessed which was subsequently used to match MOB and non-MOB partners together. In a second approach, described in Chapter 4 , "unsupervised" partner selection was accomplished by a competition experiment amongst of non-MOB partners in the methanotrophic *interactome*.

PART 3: PRESERVATION AND LONG TERM STORAGE OF *INTERACTOMES*

In Chapter 5 an optimized method for cryopreservation of (methanotrophic) *interactomes* is described, which could allow adequate preservation of optimal *interactomes* for possible biotechnological applications.

PART 4: DISCUSSION AND PERSPECTIVES FOR FUTURE RESEARCH

Finally, the combined findings over all chapters are summarized in Chapter 6. Similarities among all chapters and their significance in the light of known literature are discussed. In Chapter 7 some experiments and strategies for future research in methanotrophic *interactomes* are suggested.

The research chapters in part 2 and 3 directly fit within the CMET synthetic ecosystem flowchart, as outlined in Figure 1-10.

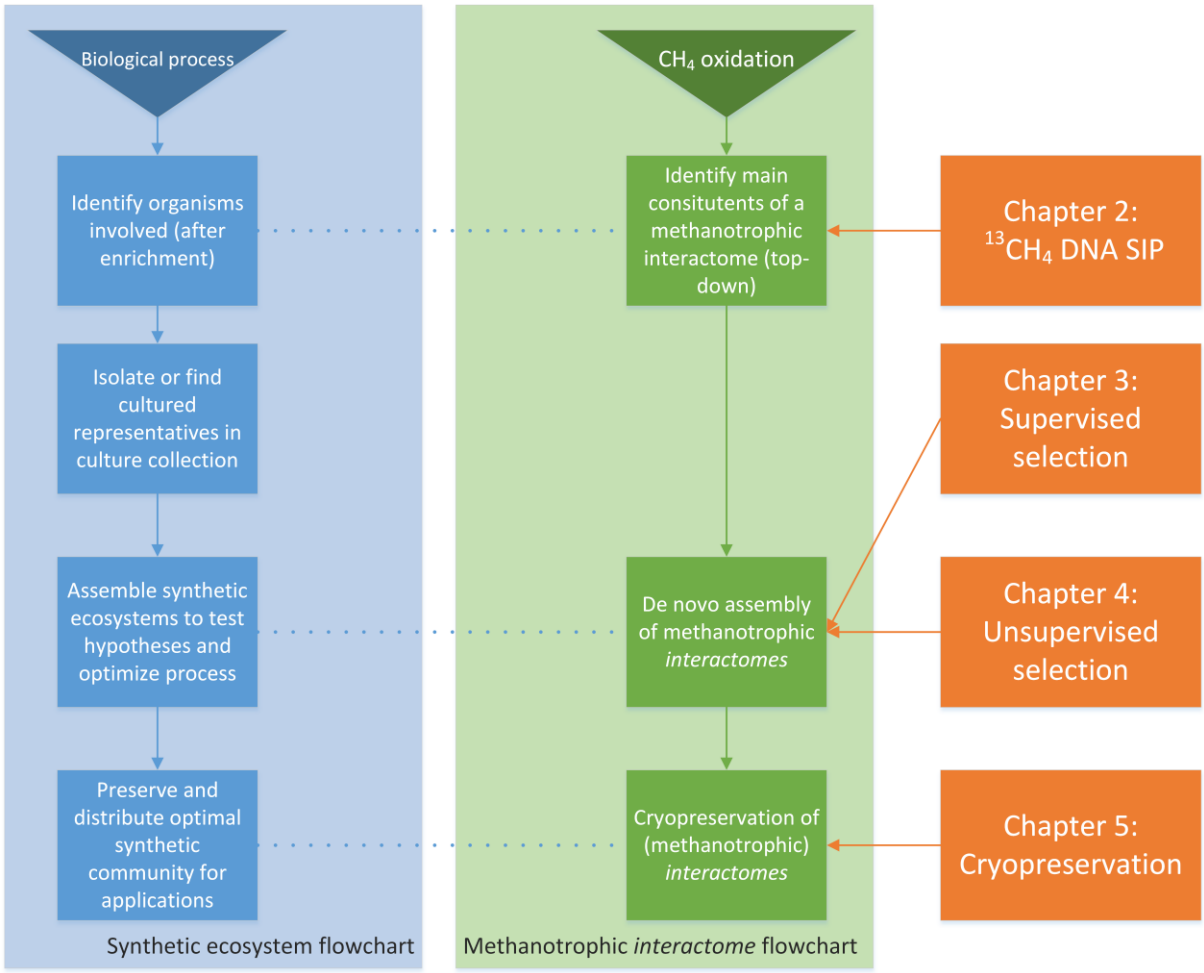
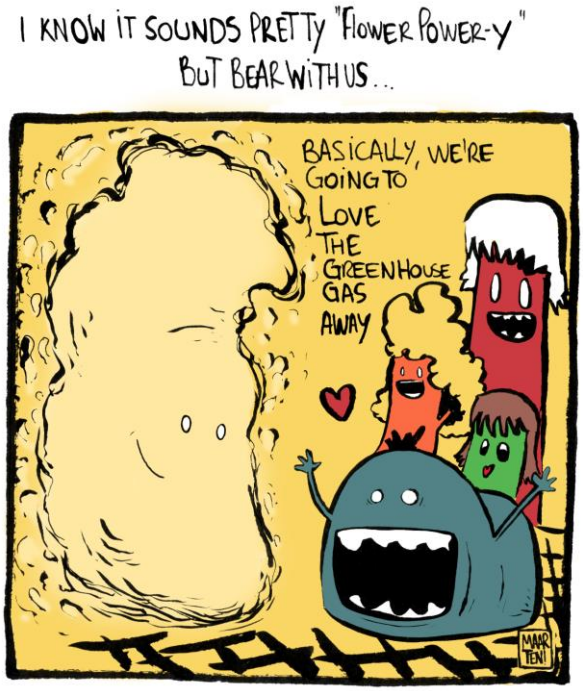


Figure 1-10. Positioning of the research chapters within the generic CMET synthetic ecosystems flowchart.

CHAPTER

2

THE METHANOTROPHIC "*INTERACTOME*": CARBON FLOW THROUGH AN INTERACTING METHANE-OXIDIZING COMMUNITY



CHAPTER 2

THE METHANOTROPHIC '*INTERACTOME*': CARBON FLOW THROUGH AN INTERACTING METHANE-OXIDIZING COMMUNITY

Abstract

Methane (CH_4) is an important greenhouse gas and one strategy for methane mitigation could rely on the use of methanotrophic bacteria (MOB). Recently, accumulating evidence demonstrated that methane oxidation is stimulated when MOB are interacting with non-methanotrophic microbes. Dissecting such an "*interactome*" can be done in a multitude of ways, of which stable-isotope probing (SIP) of the DNA is a valuable technique to track carbon flow through all partners actively involved. While most studies employing DNA-SIP to the methanotrophic *interactome* focused on the MOB that form the basis of the *interactome*, here a short-term time-resolved SIP incubation was performed in order to identify the non-methanotrophic microbes most actively involved in the *interactome*. A MOB (*Methylomonas* sp.) was found to be the primary producer supplying organic carbon to the *interactome*, and having a restricted trophic interaction with *Methylotenera* spp. as primary consumers. The relationship with secondary consumers was more promiscuous. Our findings provide insight into the methane-derived carbon flow through a methanotrophic *interactome* by focusing on the most intricately involved non-MOB community members. While confirming the previously reported link between *Methylococcaceae* and *Methylophilaceae* the diversity of secondary consumers (with *Flavobacteria, Hydrogenophaga* and *Chitinophagaceae* among the most prominent of them) is an interesting connection within the *interactome*, indicating the importance of specific biotic interactions to aerobic methane oxidation.

Chapter redrafted after:

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1. Introduction

Methane (CH_4) is an important greenhouse gas, and the majority (ca. 60%) of its emission originated from anthropogenic sources (Kirschke, Bousquet *et al.* 2013). Recently, the Intergovernmental Panel on Climate Change (IPCC) increased the global warming potential of methane from 25 to 34, using a time horizon of 100 years with inclusion of climate-carbon feedback (Myhre, Shindell *et al.* 2013). One strategy for methane mitigation could rely on the use of methanotrophic bacteria (methanotrophs, MOB). MOB are characterized by their unique ability to use CH_4 as a sole carbon and energy source (Hanson and Hanson 1996). The majority of the cultured aerobic MOB can be classified within the clades of Alpha- and Gamma-proteobacteria (Semrau, DiSpirito *et al.* 2010). These lineages can be differentiated based on their physiology, which may reflect their adopted life strategies (Ho, Kerckhof *et al.* 2013). While axenic MOB cultures are known to have many potential biotechnological applications (reviewed by Strong, Xie *et al.* (2015)), recently, accumulating evidence demonstrated that methane oxidation is stimulated when MOB are interacting with non-methanotrophic microbes (Dunfield, Liesack *et al.* 1999; Ho, de Roy *et al.* 2014; Jeong, Cho *et al.* 2014). The importance of these interactions to the methanotrophic microbiome (i.e. the methanotrophic "*interactome*") is supported by the frequent co-purification of non-MOB in methanotrophic enrichments from the environment (Lamb and Garver 1980; Holmes, Owens *et al.* 1995; Koch, Gich *et al.* 2008; Dedysh, Kulichevskaya *et al.* 2012; Takeuchi, Kamagata *et al.* 2014). These interactions can be very specific (Iguchi, Yurimoto *et al.* 2011; Stock, Hoefman *et al.* 2013), although it is not yet entirely elucidated what the determining factors to a successful partnership are. Hence, it seems that a methanotrophic *interactome* is required for effective biological aerobic methane oxidation, rather than individual obligate methanotrophic bacteria (Oshkin, Beck *et al.* 2015). Exploitation and engineering of these *interactomes* could lead to improved and sustainable mitigation and recovery of CH_4 in the form of metabolic energy or CH_4-derived carbon. For example, in environmental biotechnology methanotrophic *interactomes* are relevant e.g. for denitrification (Liu, Sun *et al.* 2014) and occur naturally in wastewater treatment plants (Ho, Vlaeminck *et al.* 2013).

To be able to engineer the methanotrophic *interactome*, insight into the active partners involved with methane assimilation is required. Stable isotope probing (SIP) is a method which enables direct linking of the active community members in an *interactome* to methane oxidation and assimilation of ^{13}C-labeled CH_4 (Dumont and Murrell 2005; Neufeld, Wagner *et al.* 2007). SIP has been employed repeatedly to investigate active microbial partners of the methanotrophic *interactome* in the environment. However, these studies focused almost exclusively on the MOB rather than the non-methanotrophic partners and are generally performed over longer timeframes, which may confound the detection of the partners most intricately involved with methane metabolism (Table 2-1).

Table 2-1. Overview of $^{13}CH_4$ - SIP experiments and their corresponding sampling times.

Reference	Sampling time(s)	Source and remarks
Morris, Radajewski *et al.* (2002)	40 days	Peat soil microcosm incubations to identify low-affinity MOB
Radajewski, Webster *et al.* (2002)	76 days	Acidic (pH=3.5) oak forest soil
Hutchens, Radajewski *et al.* (2004)	13-14 days	Microcosms of gas-bubble floating microbial mats from Movile cave
Lin, Radajewski *et al.* (2004)	36 days	Transbaikal soda lake sediments
Mohanty, Bodelier *et al.* (2006)	50 days	Rice field & forest soils with different nitrogen fertilizer amendments
Cebron, Bodrossy *et al.* (2007)	10-19 days	Slightly acidic peat landfill cover soil
Cebron, Bodrossy *et al.* (2007)	9-35 days	Wytham soil incubation with different amendments of nutrients
Murase and Frenzel (2007)	20 days	RNA-SIP of wetland rice soil
Jensen, Neufeld *et al.* (2008)	3-49 days	Haltenpipe deep-water coral reef sediment of the coast of Norway
Kalyuzhnaya, Lapidus *et al.* (2008)	3-5 days	Lake Washington sediment

Noll, Frenzel *et al.* (2008)	1-7 days	RNA-SIP of rice paddy soil
Osaka, Ebie *et al.* (2008)	2-15 days	Activated sludge for methane-dependent denitrification
Qiu, Noll *et al.* (2008)	7 days	RNA-SIP of Pulse-labeled rice rhizosphere
Han, Chen *et al.* (2009)	24 days	Alkaline soil from a Chinese coal mine
Moussard, Stralis-Pavese *et al.* (2009)	6-14 days	Marine estuary
Qiu, Conrad *et al.* (2009)	9-20 days	Young and old rice root nodules
Antony, Kumaresan *et al.* (2010)	12 days	Saline and alkaline (soda) lake
Martineau, Whyte *et al.* (2010)	8-12 days (RT), 31-45 days (4°C)	Canadian high arctic soil
Redmond, Valentine *et al.* (2010)	3-9 days	Hydrocarbon off-shore seep sediment
(Dumont, Pommerenke *et al.* (2011); Dumont, Pommerenke *et al.* 2013)	Daily for 4 days (24h)	Lake sediment for DNA, RNA and metatranscriptomic analyses
Graef, Hestnes *et al.* (2011)	14 and 28 days	High arctic peat
He, Wooller *et al.* (2012)	212-248 days (4°C) 144-212 days (10°C) 55-74 days (21°C)	Arctic lake sediment at different incubation times
He, Wooller *et al.* (2012)	188 days	Arctic lake sediment
He, Wooller *et al.* (2012)	38-212 days	Arctic lake sediment
Sharp, Stott *et al.* (2012)	14 days	Modified double-labeled approach to detect autotrophic verrucomicrobial MOB in soil
Beck, Kalyuzhnaya *et al.* (2013)	10 to 30 days	Freshwater sediments from lake Washington with different oxygen atmosphere and nitrogen source

		amendments
Saidi-Mehrabad, He *et al.* (2013)	6 to 10 days	Water sampling from oilsands tailings ponds.
Daebeler, Bodelier *et al.* (2014)	14, 21 and 28 days	Volcanic grassland soil
Putkinen, Larmola *et al.* (2014)	57 to 90 days	Sphagnum-associated methanotrophs in boreal peatlands
Sharp, Martinez-Lorenzo *et al.* (2014)	Within one week (5-7 days)	Geothermal spring soil incubated at corresponding environmental temperatures
Zheng, Huang *et al.* (2014)	19 days	Rice paddy soil

Recently, synthetic ecology approaches (Stenuit and Agathos 2015) revealed the relevance and specificity of the interactions between the MOB and non-MOB partners by assembling them together in synthetic communities (Stock, Hoefman *et al.* 2013; Ho, de Roy *et al.* 2014). Even though the exact nature of these partnerships remains to be elucidated, certain trophic interactions other than grazing or predation (Morris, Radajewski *et al.* 2002; Hutchens, Radajewski *et al.* 2004; Murase and Frenzel 2007) could be observed: as methane-derived CO_2 is readily available as the catabolic end-product of CH_4 oxidation, its autotrophic fixation (by photosynthesis) has been shown before to be an effective mode of interaction with mosses (Raghoebarsing, Smolders *et al.* 2005; Kip, van Winden *et al.* 2010) and micro-algae (van der Ha, Bundervoet *et al.* 2011; van der Ha, Nachtergaele *et al.* 2012). Furthermore, it has been known for a long time that MOB can accumulate methanol in their medium hence enabling methylotrophic organisms to grow (Wilkinson, Topiwala *et al.* 1974; Hanson 1980; Megraw and Knowles 1989), which in turn alleviate self-inhibitory effects for the MOB. Over time, other evidence has emerged that possibly, other low molecular weight carbon compounds may be metabolites released by the MOB supporting partnerships with obligate or restricted facultative methylotrophs (C1) and other heterotrophs (Figure 2-1), including facultative methylotrophs (C2 and up) (Hanson and Hanson 1996; Hrsak and Begonja 2000; Nercessian, Bienvenu *et al.* 2005; Modin, Fukushi *et al.* 2007; He, Wooller *et al.* 2012). Among these low molecular weight (LMW) carbon compounds are C1 compounds such as formic acid and formaldehyde as well as C2 up to C6 compounds such as acetate (Costa, Dijkema *et al.* 2000), succinate, hydroxybutyrate and lactate (under micro-aerobic conditions; Kalyuzhnaya, Yang

et al. (2013)), citrate (by transformation of CH_3OH initially formed by the MOB itself to citrate; Rhee and Fuhs (1978)) and sucrose (by extremophile and extremotolerant MOB (Trotsenko and Khmelenina 2002; Medvedkova, Khmelenina *et al.* 2007; But, Rozova *et al.* 2012)). Next to simple, C1 to C6 metabolites, MOB are known to produce extracellular polymeric substances (EPS), probably as a response to excess carbon as opposed to other nutrients or to oxygen stress (Hilger, Cranford *et al.* 2000; Wilshusen, Hettiaratchi *et al.* 2004; Wei, Su *et al.* 2015). As opposed to CH_4 metabolism-linked "leakage" of LMW carbon compounds and EPS, growth-dependent lysis of MOB could release protein and nucleic acids into the environment, enabling necrotrophic growth of their partners (Linton and Buckee 1977; Eisentraeger, Klag *et al.* 2001).

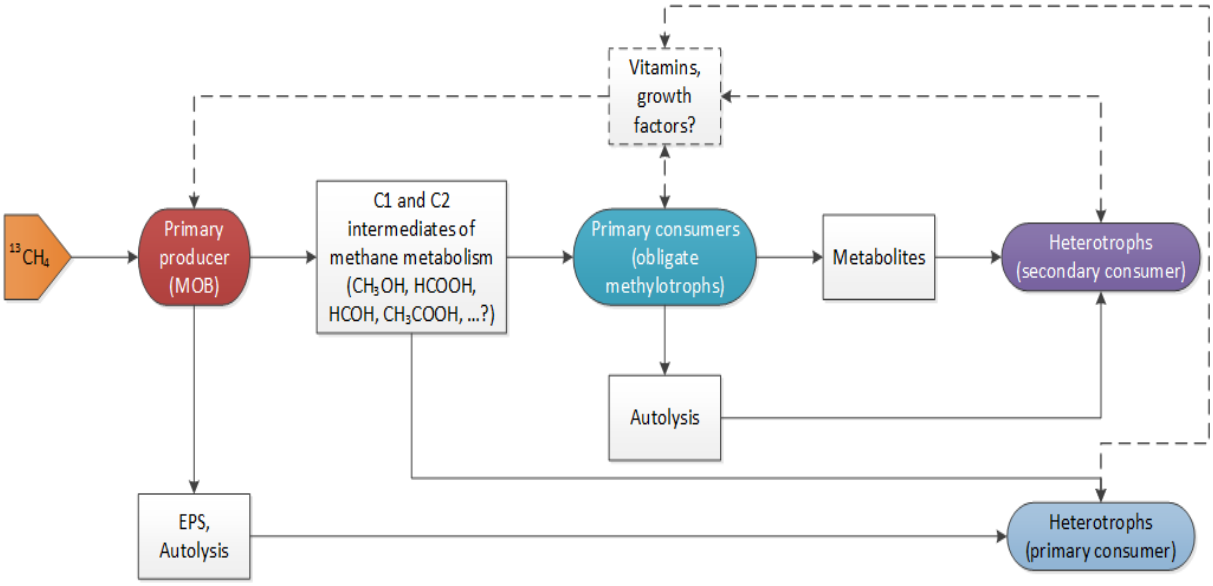

Figure 2-1. Possible interactions in the methanotrophic interactome. *In a closed methane-driven system, the methanotrophic partner is the primary producer of organic carbon for the entire interactome. Primary consumers are in a direct trophic interaction with the organic carbon produced by the MOB. This is either by consuming LMW intermediates of methane metabolism or by feeding on more complex organic molecules such as EPS or autolysis products. Secondary partners consume methane derived carbon through metabolites released by the primary consumers or by feeding on lysed primary consumers. Solid arrows represent methane-derived carbon flow through the community. Dashed arrows represent a possible flow of growth factors and vitamins of the primary and secondary consumers to the MOB.*

Evidence from synthetic ecology experiments and previous SIP and non-SIP studies suggested that preferred partnerships between MOB and the non-methanotrophic community members are in fact governing the methanotrophic *interactome* composition (Stock, Hoefman *et al.* 2013; van der Ha, Vanwonterghem *et al.* 2013). These preferred partnerships have not

been studied to a great extent, but are evidenced by co-occurrence of specific microbial groups with MOB and their assimilation of $^{13}CH_4$-derived carbon. In that respect it is important to bring into account that these partnerships are not a one-way carbon flow from the MOB to the non-MOB partners: non-MOB partners may supply growth factors to the MOB, such as proteins and vitamins (Figure 2-1). For instance, Iguchi, Yurimoto *et al.* (2011) showed that cobalamines (Vitamin B12) excreted by Rhizobia stimulated methanotrophic growth in co-cultures. The partners may also enable the MOB to survive in an environment with organic pollutants that would otherwise be toxic (Hrsak and Begonja 2000; Hesselsoe, Boysen *et al.* 2005). A metadata analysis of the 303 described partnerships of methanotrophic *interactomes* (Figure 1-8) highlighted the recurrence of specific bacterial taxa as methanotrophic microbiome partners. The most frequently described partners are known non-methanotrophic methylotrophs *Methylophilaceae* (14%, Figure 1-8) and *Hyphomicrobiaceae* (8%, Figure 1-8) which are believed to grow on C1-metabolites released by the MOB, thereby possibly alleviating toxicity effects of these compounds (Hanson and Hanson 1996). However, members of other families were also frequently identified as partners of the methanotrophic *interactome*, for example members of *Flavobacteriaceae* (5%, mainly *Flavobacterium*), *Xanthomonadaceae* (5%) and *Comammonadaceae* (6%). As stated in the previous paragraph, these microbes could encompass non-methanotrophic (facultative) methylotrophs, given the widespread phylogeny of methylotrophy (Chistoserdova, Lapidus *et al.* 2007; Kolb 2009; Chistoserdova 2011; Hung, Wade *et al.* 2011) or non-methylotrophic heterotrophs, which feed on more complex substrates (Figure 2-1). A few members of bacterial predatory families were also described frequently, however it is yet unclear in which way bacterial predation influences the methanotrophic *interactome* (Figure 1-8, Murase and Frenzel (2008)).

Given the recent evidence supporting the importance of these interactions for aerobic methanotrophy, a long-term enriched methane-driven *interactome* (van der Ha, Hoefman *et al.* 2010) was used to identify the primary producers (i.e. MOB, Figure 2-1) as well as the carbon flow pattern to the primary- and secondary consumers of the *interactome* (Figure 2-1), which have not been the focus of SIP studies up till now. To that end, DNA-SIP at a fine time resolution was combined with bidirectional pyrosequencing of the 16S rRNA gene to increase taxonomic resolution. The 16S rRNA community structure was dissected based upon richness and evenness assessments as well as indicator species analysis, to reveal which *interactome* partners are most intricately involved in $^{13}CH_4$ derived carbon assimilation.

2. Material and methods

2.1. Mixed culture growth conditions and MOR calculation

A methanotrophic enrichment was sub-cultivated over four years from the original enrichment culture from different pooled sources (van der Ha, Hoefman *et al.* 2010) in nitrate mineral salts (NMS) medium with copper (Bowman 2006) and an initial headspace concentration of 20% (v/v) CH_4. The headspace was replenished every three days. Biomass was sampled from these communities growing in active methane oxidizing fed-batch reactors and inoculated at 10% (v/v) in NMS at the start of the experiment. Batch incubations with either 20% (v/v) $^{12}CH_4$ in air (Alphagaz 2, Air Liquide, Belgium) or 20% (v/v) $^{13}CH_4$ in air (Campro Scientific Gmbh, The Netherlands) were performed in 120 mL opaque serum bottles (2*6 for the labeled, and 1*6 for the unlabeled incubation) capped with butyl rubber stoppers. The working volume was 20 mL. The bottles were incubated on a rotary shaker at 120 rpm for 92 hours in a temperature controlled room at 21 °C. Headspace gas composition (%CH_4, %O_2, %CO_2) and pressure were sampled concurrently with biomass sampling at 5, 24, 32, 60 and 92 hours. For each time point, a different serum bottle was used, as the total biomass was sampled for DNA extraction and DNA-SIP (destructive sampling). The headspace gas was sampled and its composition determined using a Compact GC® (Global Analyser Solutions, the Netherlands) equipped with a PoraBOND Q pre-column (Agilent, USA), a Molsieve 5A column and a thermal conductivity detector. The system was controlled by EZChrom Elite software (Agilent, USA). The methane oxidation rate (MOR) was calculated by means of linear regression of the methane removal profile.

2.2. DNA extraction and DNA-SIP

To track how $^{13}CH_4$ – derived carbon was assimilated by the different partners in the methanotrophic *interactome*, DNA-SIP was executed according to the protocol described by Neufeld, Vohra *et al.* (2007) for the gradient setup without EtBr. Three series of 6 serum bottles (one for each time point) were incubated with either $^{12}CH_4$ (1 series) or $^{13}CH_4$ (2 series). At each time point, the whole cultivation medium along with the MOB cells was centrifuged (8000×g, 15 minutes, 4°C) and the pellet was stored at -20 °C until further use. Sacrificial sampling was performed at relatively short time intervals (with respect to Table 2-1), to selectively detect the *interactome* partners most intricately involved in CH_4 metabolism. Only one of the $^{13}CH_4$ incubation series was analysed further and compared to

the $^{12}CH_4$ incubation series. DNA was extracted with the FastDNA® SPIN Kit (MP Biomedicals, USA) according to the manufacturer's instructions. DNA concentrations and purity were assessed with a nanodrop ND-1000 (Thermo Fisher Scientific, USA). Density of the DNA/CsCl gradient was measured using a DSA 5000M density meter (Anton Paar, Austria). Ultracentrifugation was performed using a Beckman L7-55 ultracentrifuge with a VTi 65.1 rotor (Beckman Coulter) for 40 hours at 177000×g and 20°C under vacuum. Amplification of the 16S rRNA gene from each fraction as visualized by DGGE clearly confirmed that the ^{13}C fraction was enriched for specific organisms, not occurring in the ^{12}C control incubation (Figure 2-5).

2.3. Abundance of MOB

Quantitative PCR (qPCR) assays were performed on DNA extract of the $^{12}CH_4$ and $^{13}CH_4$ incubations to measure the abundance of MOB, using the *pmoA* gene as proxy for the total MOB community. The *pmoA* gene, encoding for a subunit of the particulate methane monooxygenase enzyme, is present in virtually all obligate methanotrophs and is congruent with the 16S rRNA gene phylogeny (Knief 2015), making it a suitable biomarker for methanotrophs (Ho, Lüke *et al.* 2011). The qPCR assays specifically targeting the aerobic Alphaproteobacterial (TYPEII assay) and gammaproteobacterial (MBAC and MCOC assays) MOB were performed using the primer combinations, primer concentrations, and PCR thermal profile as described in detail by Ho, Vlaeminck *et al.* (2013). Briefly, each qPCR reaction (total volume 20 µl) consisted of 10 µl 2X SensiFAST SYBR (BIOLINE, the Netherlands), 3.5 µl of forward and reverse primers, 1 µl Bovine Serum Albumin (5 mg/ml; Invitrogen, the Netherlands), and 5 µl of 10X diluted template DNA to obtain the optimal target yield. For all assays, the melt curve was obtained from 70°C to 99°C (1°C temperature increase on each cycle). Efficiency of the qPCR reactions was within the range of 97-105%, with the slope of the standard curve of 3.2-3.4 and $R^2 > 0.98$. The qPCR was performed with a Rotor-Gene Q real-time PCR cycler (Qiagen, the Netherlands) in duplicate for each DNA extract (the ^{12}C and ^{13}C incubations) giving a total of four replicated measurements per sampling point.

2.4. PCR-DGGE and 454 amplicon sequencing

2.4.1. PCR and DGGE analysis

The 16S rRNA gene region was amplified using primer set p338F / 518R (5'-ACT CCT ACG GGA GGC AGC AG-3' / 5'-ATT ACC GCG GCT GCT GG-3') targeting the V3 variable region (Muyzer, Dewaal *et al.* 1993; Ovreas, Forney *et al.* 1997). A GC clamp of 40 bp (Muyzer, Dewaal *et al.* 1993; Ovreas, Forney *et al.* 1997) was added to the forward primer. The PCR program consisted of 10 min 95°C; 35 cycles of 1 min. 94°C, 1 min. of 53°C, 2 min. of 72°C; and a final elongation for 10 min. at 72°C. Amplification products were analysed by electrophoresis in 1.5% (wt/vol) agarose gels stained with ethidium bromide. DGGE (Denaturing Gradient Gel Electrophoresis) based on the protocol of Muyzer *et al*. (Muyzer, Dewaal *et al.* 1993) was performed using the INGENYphorU System (Ingeny International BV, The Netherlands). PCR fragments were loaded onto 8% (w/v) polyacrylamide gels in 1 × TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). To process and compare the different gels, a marker of different PCR fragments was loaded on each gel (Boon, De Windt *et al.* 2002). The polyacrylamide gels were made with denaturing gradients ranging from 40% to 60% (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run for 16 hours at 60°C and 120V. Staining and analysis of the gels was performed as described previously (Boon, Goris *et al.* 2000). The normalization and analysis of DGGE gel patterns was done with the BioNumerics software 5.10 (Applied Maths, Sint-Martens-Latem, Belgium). Ecological microbial resource management (MRM) parameters (Range-weighted richness and Gini coefficient) were calculated as described by Marzorati and colleagues (Marzorati, Wittebolle *et al.* 2008; Read, Marzorati *et al.* 2011).

2.4.2. PCR and 454 amplicon pyrosequencing

Amplicon pyrosequencing of the 16S rRNA gene was performed using a 454 XL+ Titanium system (Roche, Penzberg, Germany) as described before (Pilloni, Granitsiotis *et al.* 2012). Barcoded amplicons for multiplexing were prepared using the Ba27F/Ba519R primers (5'-AGA GTT TGA TCM TGG CTC AG-3'/5'- TAT TAC CGC GGC KGC TG-3'), amplifying the V1, V2 and V3 region of the 16S rRNA gene, extended with the respective A or B adapters, key sequence and multiplex identifiers (MID) as recommended by the manufacturer. Pyrotag PCR was performed in a Mastercycler ep gradient (Eppendorf, Hamburg, Germany) with the following cycling conditions: initial denaturation (94°C, 5 min), followed by 28

cycles of denaturation, annealing and elongation (94°C - 30s, 52°C - 30s and 70°C - 60s), followed by a final elongation (5 min - 70°C). For each sample the PCR reaction was performed in triplicate, in a final volume of 50 µl containing 1x PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 1.25 U recombinant Taq polymerase (Fermentas, St. Leon-Rot, Germany), 0.2 µg ml^{-1} bovine serum albumin (Roche), 0.3 mM of each MID-primer (Biomers, Ulm, Germany) and approximately 50 ng of template DNA. The triplicate amplicons were pooled and purified using PCRExtract Mini kit (5 PRIME, Hilden, Germany) following the manufacturer instructions. Libraries were quantified by the Quant-iT PicoGreen dsDNA quantification kit (Invitrogen, Paisley, UK), diluted accordingly and pooled in an equimolar ratio of 10^9 molecules ml^{-1}. Emulsion PCR, emulsion breaking and sequencing were performed by applying the GS FLX Titanium chemistry following supplier protocols. Pyrosequencing was performed in a Picotiter Plate, in a pool with other samples, with 26 samples per quarter of a plate.

The sequence run was performed bidirectional. The obtained reads were mapped into contigs using DNA Star Lasergenes 8 followed by Uchime chimera removal and OTU clustering at 97% using Mothur (Schloss, Westcott *et al.* 2009; Schloss, Gevers *et al.* 2011). The initial contig read were mapped to OTUs using the R language for statistical computing, version 3.2.2 (R Development Core Team 2015). This resulted in a mapping of a total of 30218 sequences into 348 overlapping "contigs" which clustered into 112 OTUs at the 97% sequence similarity cutoff among all fractions with a read length of 479 (±20) nucleotides (average ± standard deviation). The sequences were deposited in NCBI under the accession number SRP071018.

2.5. Data analysis

Representative sequences for each OTU were classified using the Naïve Bayesian Classifier as implemented in Mothur. The confidence threshold was set at 65%. Each phylogenetic taxonomy outline and template supplied on the Mothur taxonomy outline page (RDP, Silva, Greengenes) was run, followed by a custom-made R script to create a consensus taxonomy with manual correction. In case the taxonomies were not in agreement, the classification with the highest confidence score was chosen. In case all non-agreeing classifications had the same confidence score the Greengenes template and taxonomy were given preference, unless they were (part of) recommended taxonomies from Greengenes differing from the list of prokaryotic names with standing in nomenclature (LPSN, www.bacterio.net). In case of ties

among different classifications, the LPSN was consulted and the best NCBI nucleotide blast match against refseq RNA excluding environmental sequences was used to break the ties.

Alignments were performed against the SILVA v119 reference using Mothur. Taxonomic trees were built with RAxML 8.1.17 (Stamatakis 2006; Ott, Zola *et al.* 2010) with 1000 non parametric bootstraps with 123 as a random seed and the general time reversible (GTR) model of nucleotide substitution with the Γ model of rate heterogeneity with 4 discrete rate categories. From these trees an extended majority rule bootstrap consensus tree was constructed (Aberer, Pattengale *et al.* 2010; Pattengale, Aberer *et al.* 2011) and visualized with iToL (Letunic and Bork 2011).

To identify key interacting partners a Dufrêne-Legendre indicator species analysis (Dufrêne and Legendre 1997) was performed on the OTU tables of the entire dataset, randomly subsampled to the lowest sample-based sequence count using the *indval* function from the labdsv R package (version 1.7-0, Roberts (2015)) with 10000 iterations and a threshold of indicator value of 0.1 at 24h and 0.6 at 60h. The Dufrêne-Legendre indicator species analysis calculates an index that combines relative abundance of an OTU and its relative frequency of occurrence in the various fractions. The index is highest when all reads of an OTU are found in a group of fractions within either the heavy or the light fractions and when the OTU occurs in all fractions within these heavy or light fractions. Although the index was designed to show which species are indicative for a specific ecosystem in this case it was employed on “virtual” ecosystems of in-silico pooled light and heavy fractions at different time points during the analysis.

3. Results and discussion

3.1. Initial community composition, MOB activity and MOB abundance

A methanotrophic enrichment originating from various environmental samples (van der Ha, Hoefman *et al.* 2010) was enriched for over four years. Methane was given as only organic carbon and energy source and MOB were presumed to be the primary producers of the system. At the start of the experiment, the enrichment mainly comprised of Methylophilales (55%), Methylococcales (25%) and Burkholderiales (11%) (Figure 2-4). Additionally, some other orders were found in lower abundance such as unclassified Alphaproteobacteria related to Rickettsiales (2.6%), Flavobacteriales (1.6%) and Actinomycetales (1.5%). The abundances of other orders were below 1% and these included Rhodobacterales (0.82%),

Shingomonodales (0.72%) and Xanthomonadales (0.63%). Almost 25% of the reads in the initial inoculum were assigned at the genus level to *Methylomonas*, which was congruent with earlier estimates by clone library distributions from the same enrichment (van der Ha, Vanwonterghem *et al.* 2013). The community profile was consistent with a qPCR analysis (Figure 2-2) which demonstrated a numerical dominance of gammaproteobacterial *pmoA*, including those of *Methylomonas*. Combined sequencing and qPCR data suggested that the MOB supplied organic carbon to other members of the *interactome*, supporting the entire *interactome* in a 1:3 ratio (MOB:non-MOB) after prolonged non-aseptic enrichment. *pmoA* gene copies targeted by the MBAC qPCR assay increased exponentially during the incubation, while others (targeted by the TYPEII and MCOC assays) remained unchanged (Figure 2-2), suggesting an enrichment of MOB belonging to the type Ia subgroup and as indicated by the sequencing data, these were likely *Methylomonas* spp. In particular, MOB represented by the MCOC assay were detected in relatively low stable numbers in the $^{12}CH_4$ and ^{13}C incubations, indicating the type Ib subgroup population only had a limited activity in the methanotrophic *interactome*.

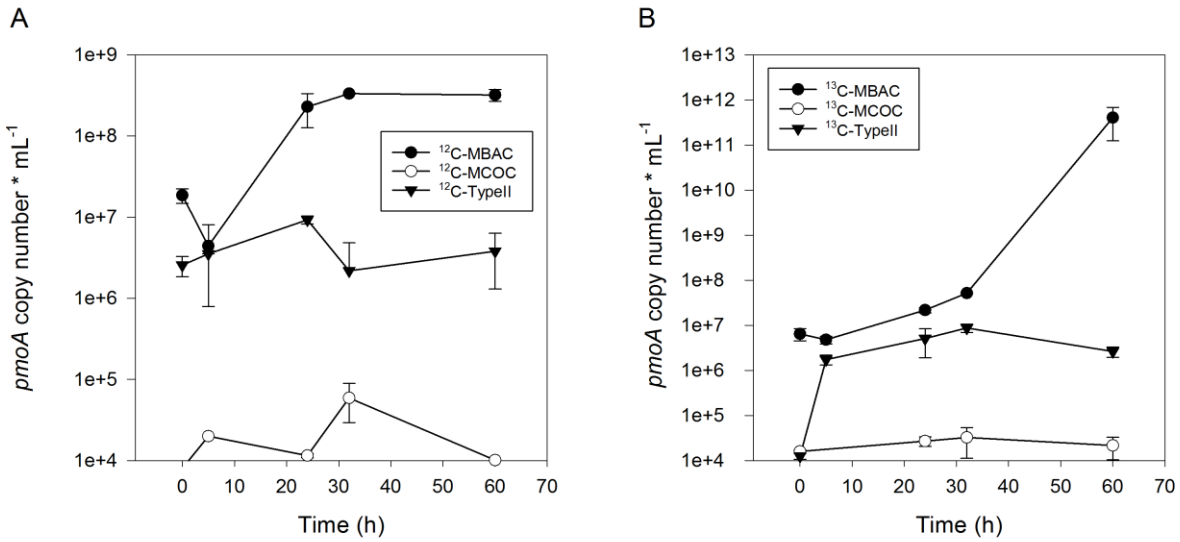

Figure 2-2. qPCR profile of the $^{12}CH_4$ and $^{13}CH_4$ incubations. *Log_{10} pmoA copy numbers for the qPCR assays as described in materials & methods (MBAC, MCOC and TypeII) are shown with the respective standard deviations (n=2) at each time point. In the case of the MCOC assay sometimes no standard deviation could be reported as one of the duplicates failed. (A) qPCR profile of the "light" incubation. (B) qPCR profile of the "heavy"incubation.*

The identification of all active partners, especially partners most intricately associated with the MOB, is essential to elucidate causal (trophic) relationships in the methanotrophic *interactome*. Thus, ^{13}C-CH_4 labeling coupled to high-throughput sequencing was applied to

identify key interacting partners by following the methane-carbon derived community composition over a high-resolution time course of 60 hours. During the experiment, the methane oxidation rate determined from the $^{12}CH_4$ and $^{13}CH_4$ incubations was comparable and averaged on 12.70 ± 3.31 mmol CH_4 oxidized per liter of culture per day (Figure 2-3).

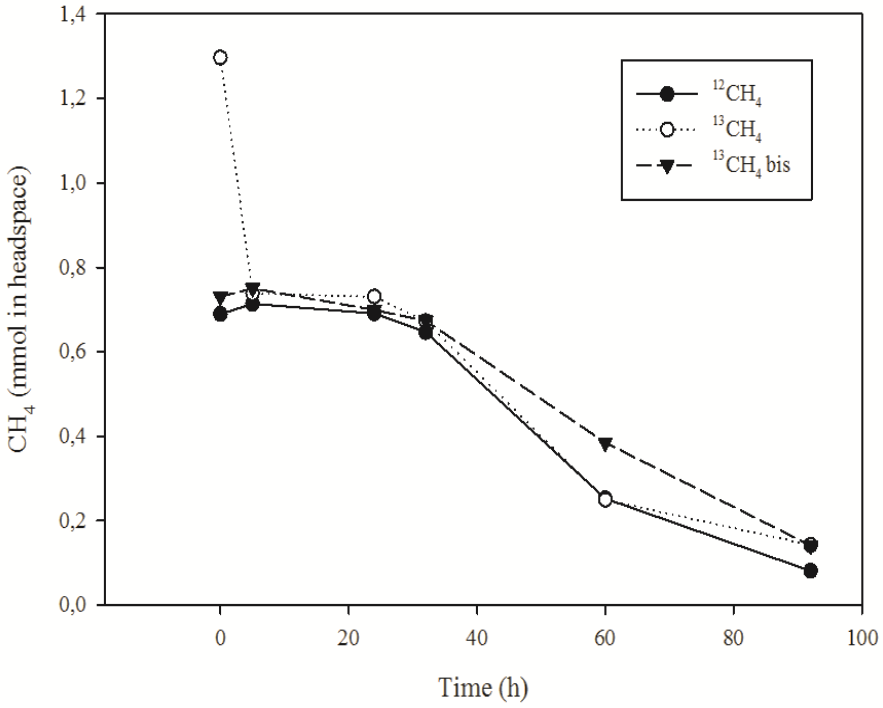

Figure 2-3. CH_4 removal profile during incubation. *At each time point an individual bottle's headspace was assessed and then sacrificial sampling was done to retrieve DNA. Given the comparable pressure of the headspace at t = 0h for $^{13}CH_4$ as compared to the other bottles, likely the amount of CH_4 in the headspace was not as high as depicted but a measurement error on the GC.*

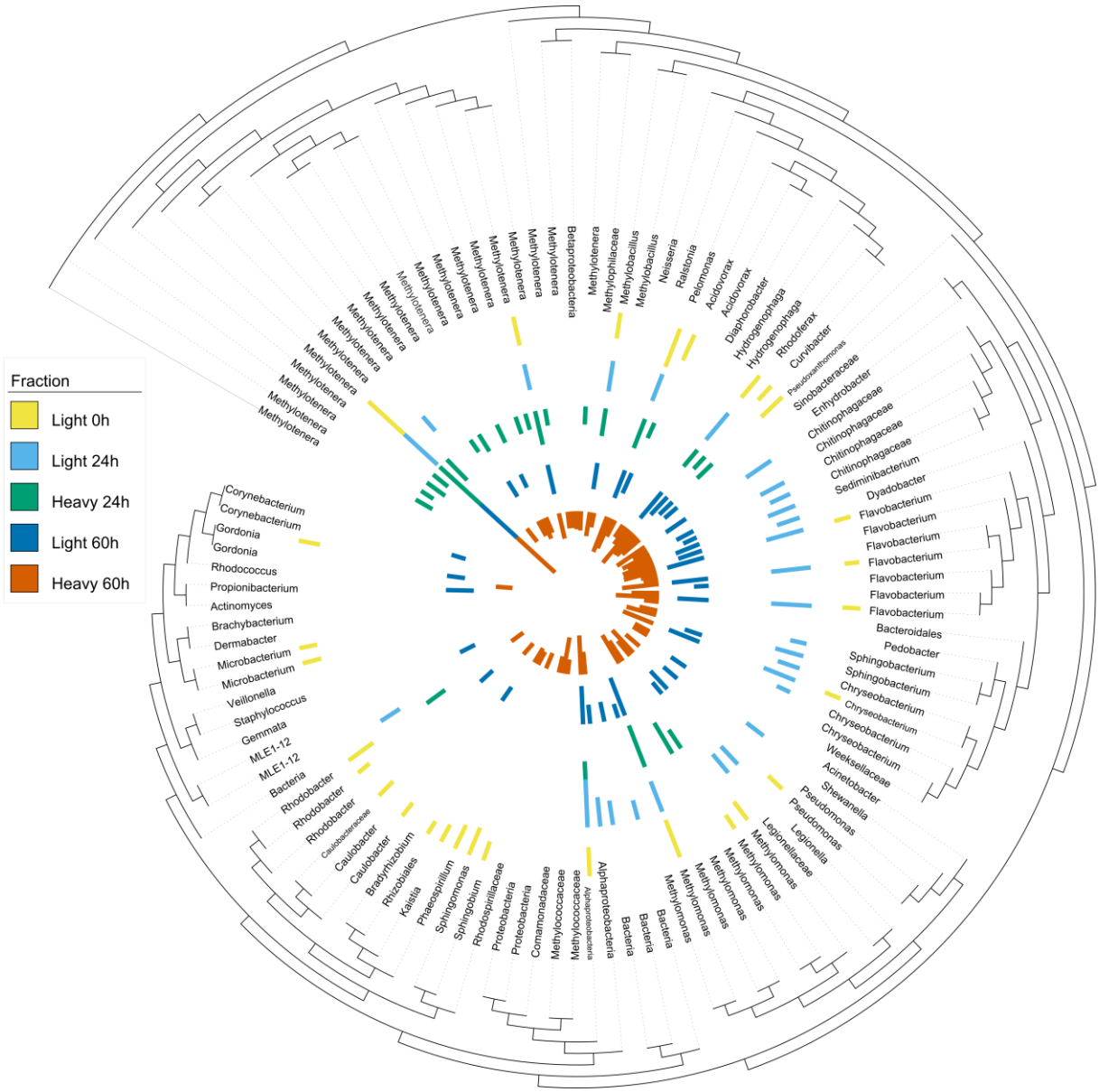

Figure 2-4. Community structure overview. *iTOL visualization of a RaXML extended majority rule consensus phylogenetic tree (GTR+GAMMA, 1000 bootstrap iterations) with log base 10 scaled sequence counts, highlighted with a separate color per sampling point. A fully interactive, zoomable, tree can be found on: http://itol.embl.de/shared/FM_Kerckhof.*

3.2. Community structure dynamics and the selection of sampling points

To follow carbon fluxes in the methane-driven *interactome* from the MOB (*Methylomonas*) to the non-MOB microbes that were most closely associated with it, sampling for SIP was done at fairly short time intervals after the start of the experiment (i.e. at 5, 24, 32 and 60 hours – compared to the sampling times in Table 2-1). An initial screening to determine when microbial community shifts occurred in the heavy fraction's 16S rRNA gene fingerprint was

performed using DGGE (Figure 2-5) by calculating two ecological indices for the heavy and light fractions at each time point.

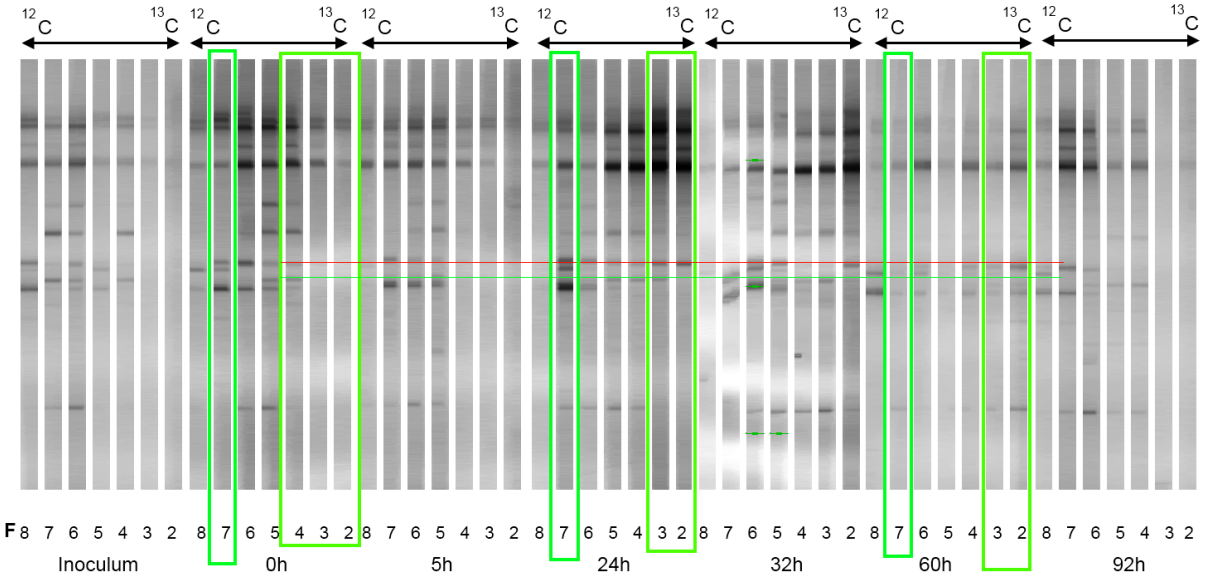

Figure 2-5. DGGE profiles of the SIP fractions. *Each fraction is indicated according to its position in the ^{12}C or ^{13}C gradient. Green boxes indicate samples that were acquired for 454 pyrosequencing. Some specific bandclasses that are popping up or disappearing from the ^{13}C fractions (and that were used to select the 454 samples) are indicated using horizontal lines.*

Firstly, the Gini coefficient (Marzorati, Wittebolle *et al.* 2008) was calculated as a measure for community evenness. Next, as a measure of community richness, we used the observed richness (S_{obs}) as determined by the band counts of our assigned band classes. As expected for a long-term enrichment, both community evenness and richness remained relatively stable (±1 observed richness, ±0.01 Gini) over the duration of the experiment for the light fraction, although an increasing trend in richness could be observed towards the end of the experiment (Figure 2-6B). For the heavy fraction, the evenness was relatively stable until 24h although the richness steadily increased before leveling off at 60h (Figure 2-6B), indicating that the active community was diversifying by inclusion of yet non-abundant primary consumers. From 32h onwards, all primary and secondary consumers of the methane-driven community became active and increased in abundance, resulting in a steep increase in evenness coinciding with an increase in richness and subsequent stabilization. Taken together, sampling at 24h and 60h was highly relevant given the dynamic community shifts, with the community diversity increasing over time in the heavy fraction. While numerous studies considered a snapshot of the active MOB community revealed by DNA-SIP, few studies followed the dynamics of the active microbial community over time with a time resolution as narrow as performed here (Table 2-1). A notable exception was the $^{13}CH_4$ time-course SIP experiment

from Lake Stechlin sediment where sampling for RNA and DNA was performed daily for 4 days (Dumont, Pommerenke *et al.* 2011), however the main focus of this research was to identify the active MOB community. Likewise Redmond, Valentine *et al.* (2010) used DNA-SIP after 72h to identify novel methane oxidizing bacteria (among others) in a marine hydrocarbon seep.

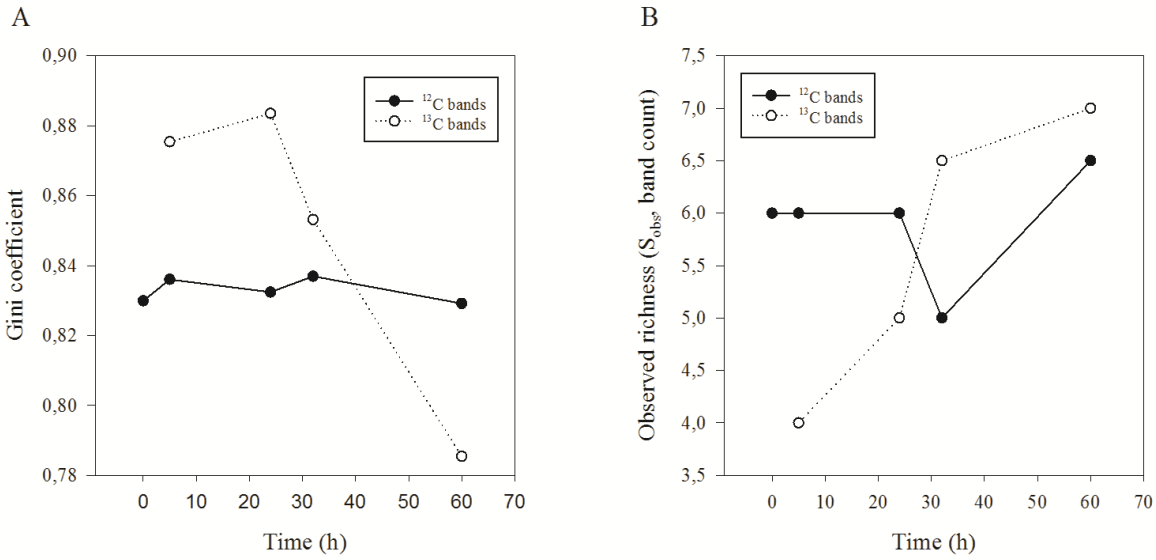

***Figure 2-6. MRM parameters** (Marzorati, Wittebolle et al. 2008) **for SIP fractions.** (A) Gini coefficient for DGGE band patterns of each of the SIP fractions (summarized in heavy and light fractions, **Figure 2-5**). A Gini coefficient of 1 represents a completely uneven community. Lower coefficients represent higher evenness. (B) Average observed richness (S_{obs}, band count) of the fractions.*

3.3. Non-MOB partners intricately associated with the methanotrophic *interactome*.

Based on the community structure metrics (Figure 2-6), time points at 24h and 60h were chosen to identify key primary and secondary consumers via 16S rRNA amplicon sequencing. The resulting OTU table was subjected to a Dufrêne-Legendre indicator species analysis (Dufrêne and Legendre 1997). The indicator value was maximal when an OTU was solely abundant within either the "heavy" or "light" fractions, and at the same time occurred in all fractions that were considered either "heavy" or "light". Hence indicative OTUs (indicative to a specific fraction at a specific time-point as a "virtual ecosystem") are not necessarily the overall most abundant OTUs, allowing indicator species analysis to circumvent possible lower incorporation of ^{13}C labels due to slower growth or indirect trophic interactions (Wintzingerode, Göbel *et al.* 1997). As expected (Figure 2-4), OTU 29 (*Methylomonas* sp.)

was found to be an indicator type ($p<0.1$) of the "virtual ecosystem" consisting of the active methanotrophic *interactome* ("heavy" fraction) during the initial 24 hours of incubation (Figure 2-7), confirming that *Methylomonas* spp. were indeed the MOB driving the methane-fueled community. Since substrate was always available in excess during incubation, the predominance of a *Methylomonas* sp. indicates that the MOB capitalized on the optimum incubation conditions, consistent with its predicted life strategy (Ho, Kerckhof *et al.* 2013). Other indicative microorganisms in the heavy fraction at 24h were all classified as *Methylotenera mobilis* (Kalyuzhnaya, Bowerman *et al.* 2006) (Figure 2-4, Figure 2-7). Given the fast (24h) incorporation in their DNA, which requires cell growth, and their described physiology as methylotrophs rather than methanotrophs, we can assume that these OTUs are representatives of primary consumers of $^{13}CH_4$ derived carbon and hence closely interacting partners of the MOB in our system (*Methylomonas* spp.). Indeed, *Methylotenera* spp. are members of the *Methylophilaceae*, a betaproteobacterial family known to be involved in C1 metabolism (Doronina, Kaparullina *et al.* 2014) with many inferred partnerships with MOB ((Beck, McTaggart *et al.* 2014; Kerckhof, Courtens *et al.* 2014) and Figure 1-8). More specifically, the association of *Methylophilaceae* with the family *Methylococcaceae* (including the genus *Methylomonas*) has already been demonstrated by Beck and colleagues for lake Washington sediments under different oxygen and nitrogen conditions (Beck, Kalyuzhnaya *et al.* 2013) for a longer incubation time (10 to 30 days) with *Methylobacter* spp. as the dominant MOB. Also, re-blasting of unclassified betaproteobacterial clones mv13.2 and mv13.9 that were shown to be associated with $^{13}CH_4$ fractions from Movile cave (Hutchens, Radajewski *et al.* 2004) showed close association with more recent NCBI refseq_rna database entries of *Methylotenera* sp. and the family *Methylophilaceae* (last accessed December 30th, 2015), which was congruent with later non-$^{13}CH_4$-SIP clone libraries picking up multiple clones identified as *Methylophilaceae* (Chen, Wu *et al.* 2009). In the Movile Cave ecosystem the most dominant MOB were also found to belong to the *Methylococcaceae*. Furthermore, He, Wooller *et al.* (2012) showed that there is a linear relationship between methylotrophs (mainly *Methylophilus* spp.) and methanotrophs (mainly *Methylobacter* and *Methylosoma*) in arctic lake sediments. Metabolic flexibility in the family *Methylophilaceae* (Lapidus, Clum *et al.* 2011) could promote co-habitation with methane-oxidizing *Methylococcaceae* without direct competition effects, and even support development of a beneficial partnership. Although the methylotrophic *alphaproteobacterial* genus *Hyphomicrobium* has repeatedly been found to co-occur with MOB (Figure 1-8), none of our OTUs could be classified as such. This may indicate a selectivity of *Methylomonas*

spp. towards *Methylophilaceae* or could be related to the specific growth conditions of our experiment.

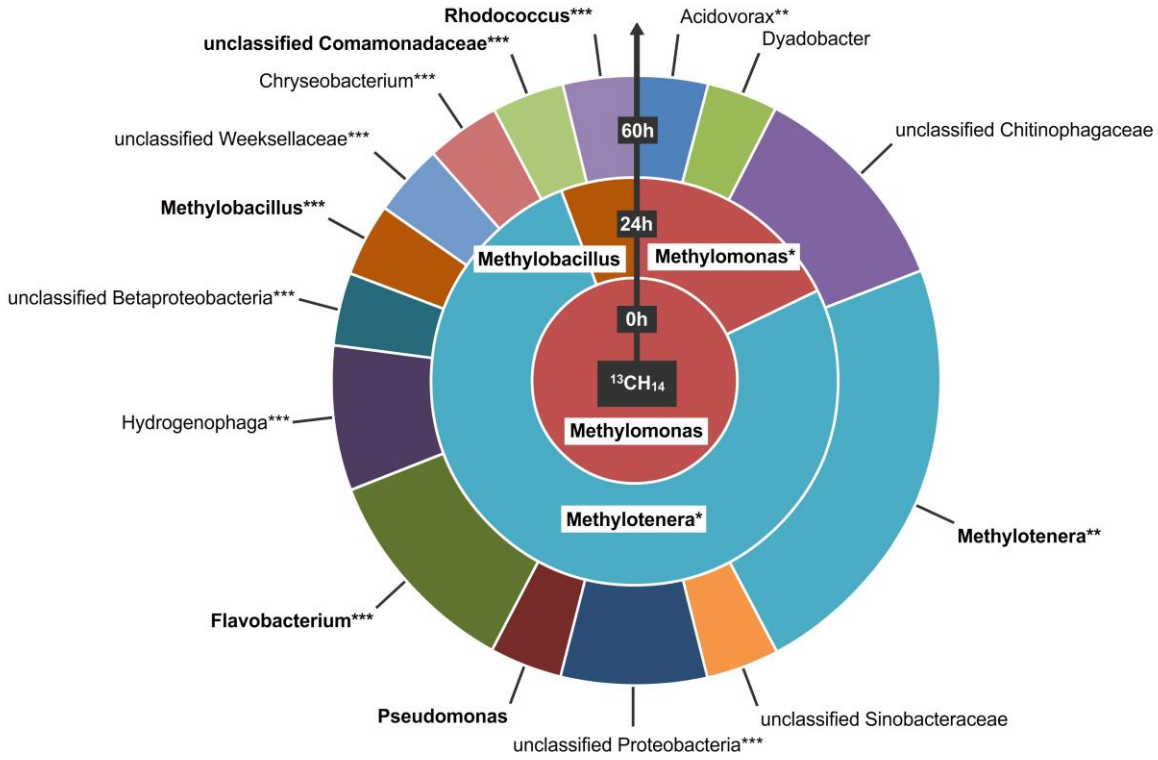

Figure 2-7. Dufrêne-Legendre indicator species analysis. *The relative amount of indicator OTUs classified with a given taxonomy at 24h and 60h of incubation (represented by the surface area). At 24h a cutoff indicator value of 0.1 was set and at 60h a cutoff indicator value of 0.6 was used. A total of 10000 random iterations were employed on a randomly subsampled (rarefied) dataset to the fraction with the least amount of reads. Methylotrophic taxa are bold faced. *: p<0.1, **: p<0.3, ***: taxon unique to 60h."Unclassified" indicates the indicative OTUs could not be classified deeper than the represented taxonomy.*

After 60 h of incubation the taxonomic diversity of indicator OTUs increased compared to 24h (Figure 2-4 and Figure 2-7), with many representatives of the phyla Betaproteobacteria and Bacteroidetes. The difference between indicative OTUs at 24h and 60h suggests rapid community dynamics of the methanotrophic *interactome*. Organisms unique to the time point 60h or enriched at 60h (for instance, the *bona fide* methylotroph *Methylotenera* spp.) were considered as secondary consumers. These secondary consumers (Figure 2-1) either thrive on dead biomass from the primary producers and primary consumers (necrotrophy) or consume secondary and more complex metabolites of the carbon assimilation pathways of the primary producers and consumers (syntrophy). Since different OTUs are associated with the *Methylotenera* spp. at 60 h as compared to 24h, the methylotrophic genus *Methylotenera* probably acted both as a primary and secondary consumer, evidencing possible intra-generic

niche differentiation (Lapidus, Clum *et al.* 2011; Hoefman, van der Ha *et al.* 2014). However, kinetics for assimilation of C1 derived carbon (such as methanol) or complex metabolites originating from MOB cell growth or decay (lysis) by *Methylotenera* spp. could differ substantially, which would bring the indicative OTUs at 60h under the denominator of "primary consumers" (Wilkinson, Topiwala *et al.* 1974). Next to indicative OTUs classified as *Methylotenera* spp., three other taxonomic groups were more representative than others for the indicative OTUs at 60h. Among those are OTUs associated with the genera *Flavobacterium* and *Hydrogenophaga* as well as the family *Chitinophagaceae*. *Flavobacteriaceae* are known to cross-feed labeled carbon in SIP experiments (4% of all reported interactions in Figure 1-8). The mode of interaction of *Flavobacteriaceae* with MOB is not conclusively elucidated: on the one hand Wilkinson, Topiwala *et al.* (1974) suggested that they remove more complex metabolites resulting from growth or lysis of the MOB, on the other hand (facultative) methylotrophic members of the genus *Flavobacterium* have been reported (Moosvi, McDonald *et al.* 2005; Madhaiyan, Poonguzhali *et al.* 2010). *Hydrogenophaga* spp. are not commonly involved with methanotrophic *interactomes* (Figure 1-8) but could perform methylotrophic metabolism (Eyice and Schäfer 2016). Finally, while *Chitinophagaceae* have been reported sporadically as $^{13}CH_4$-derived carbon assimilators (1.5%, Figure 1-8), no evidence for a methylotrophic lifestyle could be found within this family. We also identified one OTU as *Rhizobium* spp. A previous study documented a close association of MOB and members of *Rhizobium* spp., where the MOB supplied the methane-derived carbon but benefited from the seemingly mutualistic interaction with the *Rhizobium* spp. which, in return, supplied the MOB with cobalamin and other, unidentified, growth factors (Iguchi, Yurimoto *et al.* 2011). However, our indicator analysis did not show this OTU to be highly indicative to the 60h time point (Figure 2-7). Typical bacterial predators known to selectively graze on MOB are members of *Bdellovibrio* sp. (Morris, Radajewski *et al.* 2002; Hutchens, Radajewski *et al.* 2004; Murase and Frenzel 2007) but none of our OTUs were classified within this genus. Only OTU110 classified as *Vampirovibrio* sp., a non-photosynthetic Cyanobacterial obligate predatory bacterium (Soo, Woodcroft *et al.* 2015) with the microalgae *Chlorella vulgaris* as a sole prey has been identified in our study. However, the very low abundance (3 reads or 0.075%) of this OTU suggest that *Vampirovibrio* spp. were not likely to be involved in the consumption of labeled methane for the investigated timescale. Nonetheless, at a longer timescale, reclassification of clone libraries of the heavy fraction of old-nodal rice roots (Qiu, Conrad *et al.* 2009) and a methane-dependent denitrification enrichment from activated sludge (Osaka, Ebie *et al.* 2008) also

showed clones related to this *Vampirovibrio* sp. (clones O0h-89 and R1.13-27,R4.13-47), indicating that this community partner could be involved in assimilating $^{13}CH_4$ derived carbon.

3.4. Primary consumers, secondary consumers and the possible mode of interaction in the methanotrophic *interactome*

Although we did not determine the mode of trophic interaction between MOB and their partners, MOB are known to supply their partners with several forms of C1 compounds (Hanson and Hanson 1996; Costa, Dijkema *et al.* 2000; Bussmann, Rahalkar *et al.* 2006). A less-straightforward source of CH_4-derived carbon could reside in EPS (Wilshusen, Hettiaratchi *et al.* 2004), and *Methylococcaceae* (including *Methylomonas* spp.) have been reported to have a particularly high production rate of EPS (Malashenko, Pirog *et al.* 2001). Therefore, we hypothesize that the primary heterotrophic partners (Figure 2-1) are *bona fide* methylotrophs (Lidstrom 2006) capable of assimilating the readily-available C1 compounds that are released by the MOB promptly upon methane oxidation while the "secondary" partners are metabolically or kinetically less capable of metabolizing these C1 compounds. Hence, the secondary consumers have to rely on either the EPS from the MOB (in which case they can be considered primary partners), more complex metabolites from the primary partners or cell-lysis products of either (in which case they can be considered secondary partners). However, we acknowledge that our definition of primary and secondary consumers (Figure 2-1) is arbitrary as lower growth rate may just as well make actual primary consumers of the methane derived carbon only appear at 60h. Alternatively, affinity for compounds released by the MOB may be a determining factor to discriminate "primary" from "secondary" consumers. In the case of methanol it is possible that as the culture became more oxygen limited during growth (dissolved oxygen concentrations went as low as 65 µmol L^{-1}, which could be limiting depending upon the MOB strain), more methanol accumulated (Harwood and Pirt 1972) enabling methylotrophs with higher K_m values to grow which leads to an increase in the diversity of the heterotrophic partners. The indicator species analysis (Figure 2-7) indeed demonstrated that obligate or restricted facultative methylotrophs (Lidstrom (2006), further referred to as restricted methylotrophs) are distinctive to the heavy fraction at 24h while taxa known to harbor facultative methylotrophs (*Pseudomonas, Flavobacterium, Hydrogenophaga* and *Rhodococcus*) as well as non-methylotrophic taxa appear to be more indicative to the "heavy" fraction at 60h. As discussed above, presence of

facultative methylotrophs at 60h could be linked to either growth kinetics or affinity for C1 substrates, which may render these organisms less competitive as opposed to restricted methylotrophs for the same C1 carbon sources. Alternatively, the non-methylotrophic taxa indicative to 60h could point out that ^{13}C2-^{13}C(n) compounds were used as carbon source for the secondary consumers, implicating metabolic divergence between primary and secondary consumers of the methane derived carbon. Nevertheless, care should be taken when declaring a certain taxon is non-methylotrophic as facultative methylotrophy is probably phylogenetically more diverse than currently known, and may be subject to horizontal gene transfer (Boden, Thomas *et al.* 2008; Chistoserdova 2011; Hung, Wade *et al.* 2011). In summary, the distinction between primary and secondary consumers of methane-derived carbon is likely an interplay between metabolic capabilities and kinetics leading to niche differentiation of partners involved in the methanotrophic *interactome*.

4. Conclusion and perspectives

In this study, we have employed a relatively fine time-scale resolution to identify the most intricate partnerships between aerobic methanotrophic bacteria (MOB) as primary producers and other bacteria as primary and secondary consumers of methane derived carbon using stable isotope probing. *Methylomonas* was the driver of the methane-based food web and, as anticipated, the primary consumers belonged to the family *Methylophilaceae*, indicating C1-driven growth is responsible for restricted trophic relationships and thereby, re-affirming previously reported close interactions between *Methylococcaceae* and *Methylophilaceae*. As the incubation with the isotopically labeled methane progressed, the C1-derived *interactome* became more diverse indicating more promiscuous secondary consumer partnerships. This research could serve as a first step of a mechanistic understanding of these partnerships and their genetic background potentially leading to a better microbial resource management of methanotrophic *interactomes* for biotechnological applications such as micropollutant degradation (Benner, De Smet *et al.* 2015) or methane mitigation and extending concrete life (Ganendra, De Muynck *et al.* 2014; Ganendra, Mercado-Garcia *et al.* 2015). Alternatively, this understanding could help in the design of synthetic ecology experiments for *in vitro* validation of methanotrophic partnerships (Chapter 7), which could lead to biotechnological applications such as single-cell protein production. In this case, high-throughput isolation by miniaturized extinction culturing (Hoefman, van der Ha *et al.* 2012) could precede a large-scale combinatorial setup such as previously performed by Stock, Hoefman *et al.* (2013) but

with a combination of presumed primary and secondary partners (Namsaraev and Zavarzin 1972).

5. Acknowledgments and author contributions

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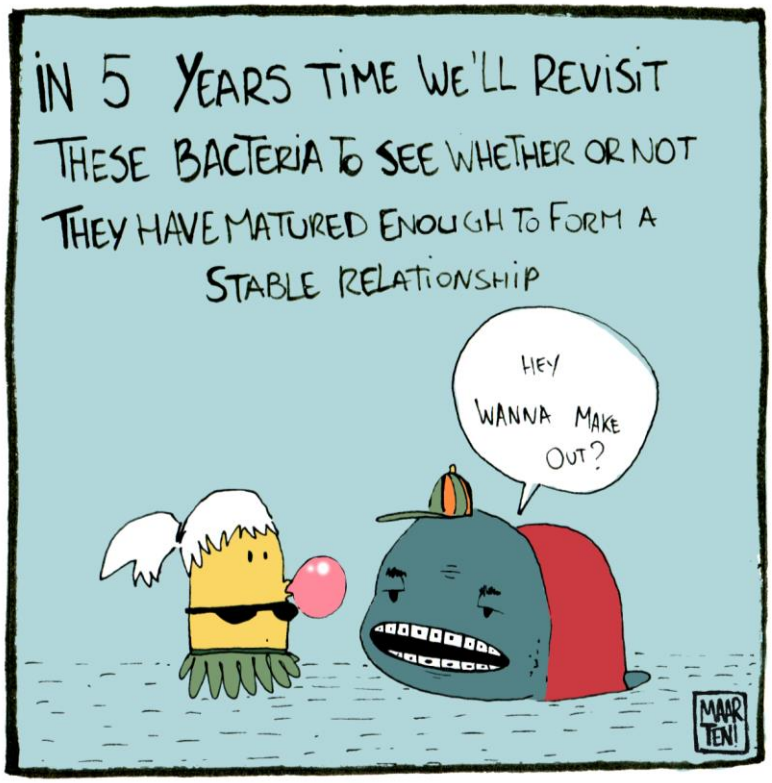
CHAPTER

3

OPTIMAL PARTNERSHIPS IN THE METHANOTROPHIC *INTERACTOME*: SUPERVISED SELECTION



CHAPTER 3

OPTIMAL PARTNERSHIPS IN THE METHANOTROPHIC *INTERACTOME*: SUPERVISED SELECTION

Abstract

Methane oxidizing bacteria (MOB) are a unique bacterial clade with the distinctive capacity to use methane (CH_4) as their sole carbon and energy source. During isolation of MOB, often non-MOB partner strains co-purify and accumulating evidence of the importance and specificity of interactions between MOB and non-MOB partners attributes to the belief that a methanotrophic *interactome* rather than axenic MOB are performing the biological oxidation of CH_4 in natural systems. We hypothesized that specific non-MOB partners are preferred by the MOB and may stimulate MOB growth and activity. This hypothesis was assessed by making 1:1 combinations of two MOB types with 38 non-MOB (i.e. 76 1:1 *interactomes*). Additionally, we hypothesized that this specificity is an "acquired taste" which allows for adaptation of the MOB and non-MOB partner. To address this hypothesis, we combined both MOB types with a fixed partner which was found to be moderately compatible during the assessment of the first hypothesis and co-cultivated it for multiple cycles. In each cycle the partnership was challenged by variable partners with differing 1:1 compatibility with the MOB and the resulting functionality (CH_4 oxidation rate, MOR) and numerical composition of the 1:1:1 *interactomes* were assessed. We found that indeed MOB were able to sustain different non-MOB partners to a specific extent, confirming the first hypothesis. Overall, an alphaproteobacterial MOB had a greater positive effect on its non-MOB partners which in turn had a greater stimulatory effect for the MOB as compared to a gammaproteobacterial MOB. In contrast to the second hypothesis, adaptation did not have a clear effect on MOR. Only limited adaption was observed for the fixed partner with the alphaproteobacterial MOB. This was reflected by the abundance of the fixed partner in the resulting *interactomes*, showing that only in very few cases the fixed partner could persist, regardless of the presence of variable partners with different initial compatibility. Overall, the alpha- and

gammaproteobacterial MOB differentially impacted the non-MOB partners and it became clear that the initial compatibility classes observed from a single cycle 1:1 *interactome* could not be extrapolated to repeated cycles of co-cultivation.

Chapter redrafted after:

Invading the methanotrophic *interactome*: partner selection regardless of initial compatibility. Kerckhof F.-M., Lacoere, T., Ho, A., Vilchez-Vargas, R., Heylen, K., Boon, N. *In preparation.*

1. Introduction

Methane oxidizing bacteria (MOB) are a unique bacterial clade with the distinctive capacity to use methane (CH_4) as their sole carbon and energy source (Hanson and Hanson 1996). While many MOB have been isolated and brought into axenic culture, metagenomic evidence suggests that many uncultured MOB are still present in the environment (Knief 2015). Additionally, during isolation of MOB, often non-MOB partner strains co-purify thwarting axenic cultivation of the MOB (Dedysh, Kulichevskaya *et al.* 2012; Takeuchi, Kamagata *et al.* 2014). This and other accumulating evidence (Stock, Hoefman *et al.* 2013; Ho, de Roy *et al.* 2014) of the importance and specificity of interactions between MOB and non-MOB partners attributes to the belief that a methanotrophic *interactome* rather than axenic MOB are performing the biological oxidation of CH_4 in natural systems (Oshkin, Beck *et al.* 2015).

Given the importance of CH_4 as a greenhouse gas (Nisbet, Dlugokencky *et al.* 2014), a thorough understanding of the mechanisms for its biological oxidation is becoming increasingly relevant. Furthermore, the methanotrophic *interactome* is an excellent model system to study bacterial interaction and adaptation: the interaction between MOB and non-MOB partners is believed to be a "facultative" mutualistic one where the non-MOB cross-feed on CH_4-C by scavenging metabolites and thereby relieving the MOB of inhibitory intermediate metabolites of CH_4 metabolism (Megraw and Knowles 1989; Hanson and Hanson 1996; Modin, Fukushi *et al.* 2010; Iguchi, Yurimoto *et al.* 2015). Some reports additionally show a mutually beneficial exchange of metabolites between the MOB and non-MOB (Lamb and Garver 1980; Iguchi, Yurimoto *et al.* 2011). Furthermore, MOB are known to exude secondary metabolites under certain conditions (e.g. sucrose, ectoine; Khmelenina, Rozova *et al.* (2015)) and methanobactin (Kim, Graham *et al.* 2004)) and produce extracellular polymeric substances (Wilshusen, Hettiaratchi *et al.* 2004). On the other hand, competition for oxygen or nitrogen sources may occur between the *interactome* partners (Hernandez, Beck *et al.* 2015). Additionally, parasitic or necrotrophic interactions have also been reported (Linton and Buckee 1977; Murase and Frenzel 2008). Conversely, our group previously reported specific associations in a co-cultivation experiment with 9 MOB and 25 non-MOB based upon the total biomass (measured as absorbance at 600 nm, OD_{600}) of 1:1 combinations relative to axenic MOB growth (Stock, Hoefman *et al.* 2013). Therefore, two hypotheses were assessed in this chapter. First, the hypothesis that specific non-MOB partners are preferred by the MOB (Hypothesis 1) and may stimulate MOB growth and activity was investigated. This hypothesis was assessed by making 1:1 combinations of two MOB types

with 38 non-MOB (i.e. 76 1:1 *interactomes*) and quantifying the abundance of both partners after 1 cycle of co-cultivation. Next, the hypothesis that this specificity is an "acquired taste" which allows for adaptation and co-evolution of the MOB and non-MOB partner was investigated (Hypothesis 2). To address this hypothesis, both MOB types were combined with a fixed partner which was found to be moderately compatible during the assessment of the first hypothesis and co-cultivated it for multiple cycles. In each cycle the partnership was challenged by variable partners with differing 1:1 compatibility with the MOB and the resulting functionality (CH_4 oxidation rate, MOR) and numerical composition of the 1:1:1 *interactomes* were assessed.

2. Material and methods

2.1. Bacterial strains, growth conditions and experimental design

A total of 38 non-MOB strains (Table 3-1) were co-cultivated with both an alphaproteobacterial (LMG 26262, *Methylosinus* sp.) and gammaproteobacterial (NCIMB 111130^T, *Methylomonas methanica*) MOB. All non-MOB strains were pre-cultivated as described previously (Stock, Hoefman *et al.* 2013), and were selected based upon the availability of their full genomes (for strains with publicly available genomes) as well as their distinct colors on CR2 medium for the Leipz 1-5 strains or good preliminary results of co-cultivations for batch incubations with Arthro. Briefly, after resuscitation from -80°C stocks, non-MOB strains were cultivated on nutrient agar (NA; Oxoid, UK) at 28°C for 24 hours except for *Rhodobacter sphaeroides* LMG 2827^T, which was pre-cultivated on tryptone soy agar (TSA; CM0131, Oxoid, UK) at 28°C for 48 hours (in the dark) and strains Leipz1 up to Leipz5 (Saleem, Fetzer *et al.* 2012) which were pre-cultivated on Brunner CR-2 medium (Saleem, Fetzer *et al.* 2012). After pre-cultivation, a single colony was picked and transferred at least once to a fresh plate with the respective agar before use in the experiment. Purity was assessed using partial 16S rRNA gene sequencing (primers 63F-1378R). MOB strains were pre-cultivated in 0.22 µm sterile filtered liquid nitrate mineral salts medium (NMS, Bowman (2006)) with Cu^{2+} adjusted to 10 µM. MOB pre-cultivation was performed at 28°C on a rotary shaker in 120 mL opaque serum bottles with a working volume of 20 mL. The serum bottles were sealed air-tight with opaque rubber stoppers after which 20 mL 0,22 µm sterile filtered CH_4 (Linde Gas, HiQ 4.5) was added, leading up to a final headspace concentration of 20%

(v/v) CH_4 in air. Purity of the MOB was checked using regular plating on 10% TSA as well as partial 16S rRNA gene sequencing (primers 63F-1378R).

Table 3-1.Strains used and their taxonomic class. *If the genus contains a described methylotroph (Lidstrom 2006; Boden, Thomas et al. 2008; Kolb 2009; Hung, Wade et al. 2011) it was reported. A distinction between obligate (facultative restricted) and facultative methylotrophs was made (Chistoserdova 2011). When possible the GOLD project ID (gold.jgi.doe.gov) is reported. Superscript T in the strain/coding column indicates the strain is the type strain of the species.*

Strain / coding	Species	Class	Known methylotrophs in genus?	Genome availability
LMG 1025	*Acinetobacter baumannii*	Gammaproteobacteria	facultative	Gp0000191
LMG 2844^T	*Aeromonas hydrophila subsp. Hydrophila*	Gammaproteobacteria	no	Gp0000262
LMG 2134^T	*Ochrobactrum anthropi*	Alphaproteobacteria	no	Gp0000090
LMG 1267^T	*Chromobacterium violaceum*	Betaproteobacteria	no	Gp0000613
LMG 1201	*Cupriavidus necator*	Betaproteobacteria	facultative	Gp0000312
LMG 7866^T	*Escherichia fergusonii*	Gammaproteobacteria	no	Gp0001407
Artrhro	*Arthrobacter sp.*	Actinobacteria	facultative	*In-house*
R-23894	*Escherichia coli*	Gammaproteobacteria	no	Gp0001423
LMG 2827^T	*Rhodobacter sphaeroides*	Alphaproteobacteria	facultative	Gp0000309
Leipz1	*Williamsia* sp.	Actinobacteria	no	*In-house*
Leipz2	*Rhodococcus* sp.	Actinobacteria	facultative	*In-house*
Leipz3	*Micrococcus* sp.	Actinobacteria	facultative	*In-house*
Leipz4	*Janthinobacterium* sp.	Betaproteobacteria	no	*In-house*
Leipz5	*Agrobacterium* sp.	Alphaproteobacteria	no	*In-house*
R-23891	*Escherichia coli*	Gammaproteobacteria	no	Gp0000745
R-23892	*Escherichia coli*	Gammaproteobacteria	no	Gp0000785
R-23895	*Escherichia coli*	Gammaproteobacteria	no	Gp0000663
LMG 1197	*Cupriavidus pinatubonensis*	Betaproteobacteria	facultative	Gp0000452
LMG 21445^T	*Burkholderia phymatum*	Betaproteobacteria	facultative	Gp0002243
LMG 1195^T	*Cupriavidus metallidurans*	Betaproteobacteria	facultative	Gp0000357
LMG 22485^T	*Burkholderia lata*	Betaproteobacteria	facultative	Gp0000435
LMG 20219^T	*Burkholderia thailandensis*	Betaproteobacteria	facultative	Gp0000407
LMG 287	*Rhizobium radiobacter*	Alphaproteobacteria	no	Gp0000707
LMG	*Sphingomonas*	Alphaproteobacteria	facultative	Gp0000387

Strain / coding	Species	Class	Known methylotrophs in genus?	Genome availability
18303^T	*aromaticivorans*			
LMG 22486	*Burkholderia vietnamiensis*	Betaproteobacteria	facultative	Gp0000181
LMG 19005^T	*Shewanella oneidensis*	Gammaproteobacteria	no	Gp0000671
LMG 17588	*Burkholderia multivorans*	Betaproteobacteria	facultative	Gp0002242
R-17801	*Pseudomonas putida*	Gammaproteobacteria	facultative	Gp0000665
R-23900	*Pseudomonas syringae*	Gammaproteobacteria	facultative	Gp0000618
R-23899	*Pseudomonas syringae subsp. syringae*	Gammaproteobacteria	facultative	Gp0000484
R-23897	*Pseudomonas fluorescens*	Gammaproteobacteria	facultative	Gp0000479
R-23898	*Pseudomonas fluorescens*	Gammaproteobacteria	facultative	Gp0000439
LMG 22487^T	*Burkholderia phytofirmans*	Betaproteobacteria	facultative	Gp0002244
LMG 24309	*Burkholderia ambifaria*	Betaproteobacteria	facultative	Gp0002240
LMG 24507	*Burkholderia cenocepacia*	Betaproteobacteria	facultative	Gp0000271
LMG 24210	*Pseudomonas putida*	Gammaproteobacteria	facultative	Gp0000136
LMG 12228	*Pseudomonas aeruginosa*	Gammaproteobacteria	facultative	Gp0000751
LMG 19847^T	*Brachybacterium faecium*	Actinobacteria	no	Gp0001925
LMG 26262 (L)	*Methylosinus* sp.	Alphaproteobacteria	Obligate, MOB	*In-house*
NCIMB 11130^T (R)	*Methylomonas methanica*	Gammaproteobacteria	Obligate, MOB	*In-house*

Subsequently, the experiment was run in two phases (Figure 3-1): in a first phase a compatibility of each non-MOB partner with each MOB was established (76 combinations). After ranking (see section 2.3 of this chapter), initial compatibility was subdivided in three compatibility classes: lowly, moderately and highly compatible non-MOB partners were distinguished. For the second phase, a fixed partner was selected from the moderately compatible partners (of both MOB), with additional consideration for the total *interactome* biomass increase (measured as OD_{600}) and effect on MOB abundance. Two variable partners were selected from each of the compatibility classes. The fixed partner was then co-cultivated for multiple cycles with the MOB, and in each cycle this 1:1 *interactome* was challenged with each of the variable partners separately (Figure 3-1).

As an inoculum for Phase 1, MOB and non-MOB were combined (in duplicate) into 1:1 *interactomes* as described previously (Stock, Hoefman *et al.* 2013). Briefly, colonies were suspended in liquid NMS to a final absorbance of 0.005 at 600 nm (OD_{600}) and then combined in liquid NMS in a sterile 96-well microtiter plate (200 µL, final working volume). Subsequently, plates were incubated at 28°C for approximately 7 days in airtight jars with a 1:1 CH_4:air atmosphere. Un-inoculated control wells were used to correct for possible changes in OD_{600} due to changes or evaporation of the NMS medium. A control plate was included in the same jar with wells inoculated only with MOB or non-MOB partners (each well only containing a single strain, unreplicated).

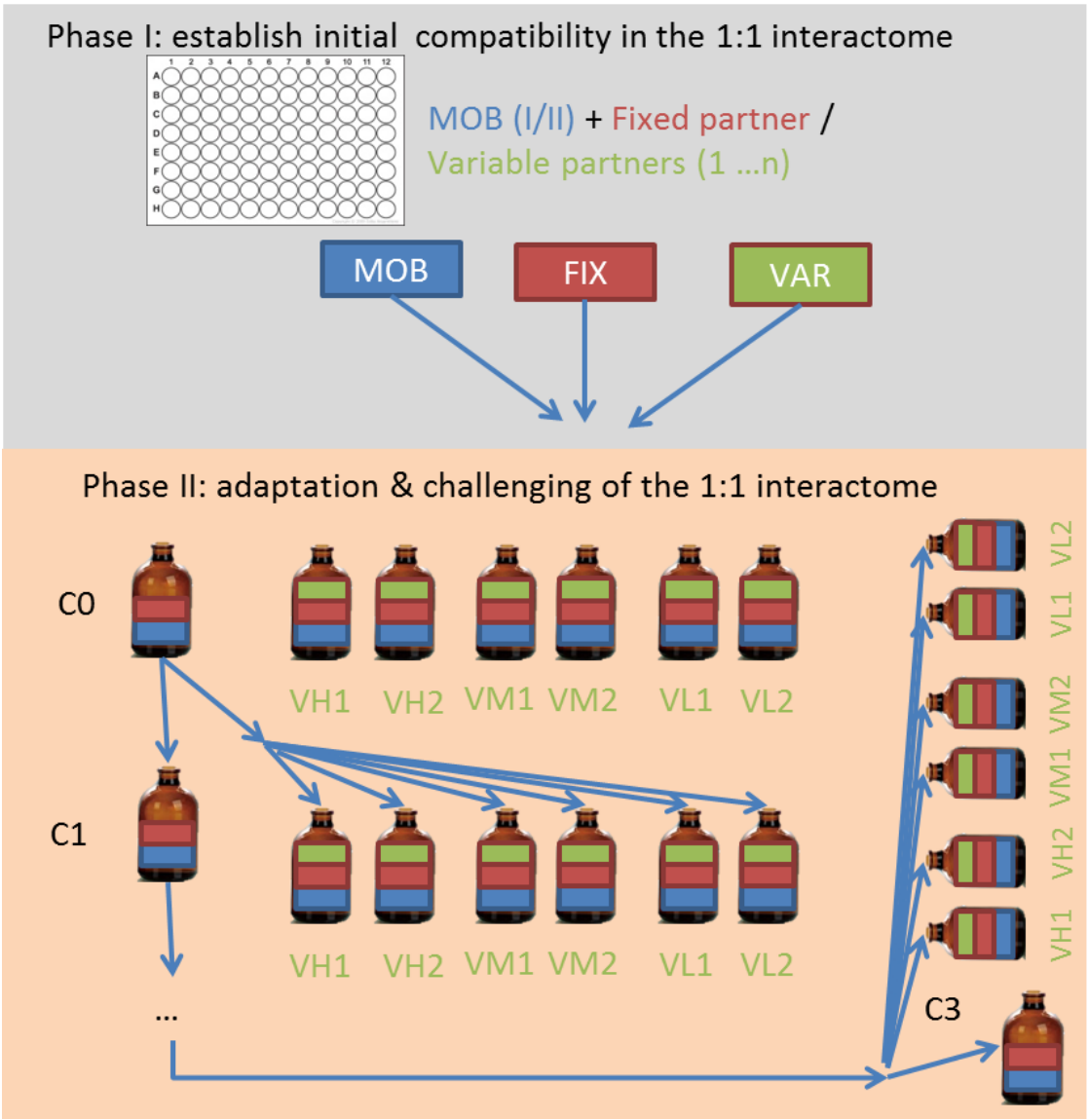


Figure 3-1. Overview of experimental design. *C0 – C3 correspond to repeated cycles of co-cultivation. VH1,VH2 refer to highly compatible variable partners 1 and 2. VM1, VM2 refer to moderately compatible partners while VL1 and VL2 refer to lowly compatible partners. In Phase I either the fixed partner or the variable partners were co-cultivated with the MOB.*

In the second phase the selected fixed and variable partners (see section 3 of this chapter), were co-cultivated in 20 mL NMS medium (with copper) (Whittenbury, Phillips *et al.* 1970; Bowman 2006) in 120 mL opaque serum bottles closed air-tight with grey butyl rubber stoppers. The bottles were incubated on a rotary shaker (120 rpm) at 28 °C. At the start of each cycle of 6 to 7 days, the headspace of the serum bottles contained 20% (v/v) of CH_4 (Linde Gas, HiQ 4.5) in air. If during incubation headspace oxygen concentrations dropped below 2% (v/v), additionally 20mL of O_2 was injected into the headspace, to avoid O_2 limitation of the co-culture. At the end of each cycle the bottles were opened under laminar flow and the liquid medium was transferred to sterile 50 mL falcon tubes. 2 mL was centrifuged at 18000 g for 15 minutes (at 4°C) and, after removal of supernatant, the pellets were stored at -20°C until DNA extraction. The rest of the culture broth with the MOB and the fixed partner or the MOB culture as such was diluted with NMS medium to OD_{600} 0.005 in great enough volumes for triplicate co-cultivation with each of the variable partners (MOB+Fix+Var) and duplicate co-cultivation without the variable partners (MOB+Fix and MOB).

2.2. Analytical methods

During Phase 2 (Figure 3-1), headspace gas and pressure were sampled daily or every other day. The headspace gas composition (CH_4, O_2, CO_2) was determined using a Compact GC® (Global Analyser Solutions, the Netherlands) equipped with a PoraBOND Q pre-column (Agilent, USA), a Molsieve 5A column and a thermal conductivity detector. The system was controlled by EZChrom Elite software (Agilent, USA). The methane oxidation rate (MOR) was calculated by means of linear regression of the methane removal profile.

Optical density at 600 nm for phase 1 and 2 were measured using a Tecan Infinite M200 pro (Tecan, Austria), with correction for the lid of the microtiter plate.

2.3. DNA extraction, primer design, qPCR and data analyses

DNA was extracted from the whole content of a well (200 µL) from Phase 1 or the pellet of 2 mL of culture from Phase 2 using a FastPrep mechanical bead-beating protocol combined with phenol/chloroform extraction as described before (Vilchez‐Vargas, Geffers *et al.* 2013). To track the individual constituents of the *interactome* specific qPCR primers were designed for the *pmoA* gene of the MOB and the 16S rRNA gene of the fixed partner (Table 3-2). The amount of variable partner in Phase 2 was calculated by subtracting a copy-number

corrected count of the MOB and fixed partner copies of a total count of eubacterial 16S rRNA gene copies. Primer design was performed using ARB (version 6.0.3, Ludwig, Strunk *et al.* (2004) in conjunction with Primer3Plus (Untergasser, Nijveen *et al.* 2007) and BioEdit. Primer specificity for the fixed partner was assessed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, Ye, Coulouris *et al.* (2012)) against the selected variable partners and using SILVA TestPrime (http://www.arb-silva.de/search/testprime/, Klindworth, Pruesse *et al.* (2013)) and RDP ProbeMatch (http://rdp.cme.msu.edu/probematch/search.jsp, Cole, Wang *et al.* (2009)) against the whole 16S rRNA gene databases.

Table 3-2. Overview of primers used in this work. *When possible, probeBase (http://probebase.csb.univie.ac.at/) accession numbers were reported.*

Primer set name (reference)	Oligo name (probeBase accession)	Oligo sequences (5' to 3')	Target/Use
Tim-typeII (this work)	317F	AGTGGATCAACCGCTACGTGAACT	LMG 26262 specific *pmoA* gene qPCR
	610R	TCGAGGTGCGGACGAAGTGGA	
Tim-methylohigh (this work)	319F	AGTGGATYAACCGTTAYATGAACT	NCIMB 11130^{T} specific *pmoA* gene qPCR
	520R	ACHGGCCAGTTRCCTGGGTAGA	
ArthroTim (this work)	190F	TGACTCCTCATCGCATGGT	Fixed partner *Arthrobacter* sp. Specific qPCR
	346R	TGGCCGGTCACCCTTTCA	
16S V3 (Ovreas, Forney *et al.* 1997)	(P)338f (pB-3826)	ACTCCTACGGGAGGCAGCAG	General bacterial 16S qPCR
	(P)518r (pB-3827)	ATTACCGCGGCTGCTGG	
16S V1-V6 (El Fantroussi, Verschuere *et al.* 1999)	(P)63f (pB-3825)	CAGGCCTAACACATGCAAGTC	Partial 16S sequencing primer for Sanger sequencing
	1378R	CGGTGTGTACAAGGCCCGGGAACG	

The PCR and qPCR thermal profiles are listed below. Annealing temperatures were optimized using thermal gradients and amplicon quality control on agarose gel.

Real-time PCR (qPCR) was performed on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Duplicate (Phase 1) and triplicate (Phase 2) samples of a 10-fold dilution of the DNA-samples were analyzed for the targets listed in Table 3-2.

The reaction mixture of 25 µL was prepared by means of the Fermentas Master Mix (Thermo Scientific) and consisted of 4.8 µL of Fermentas Master Mix, 14.1875 µL of nuclease-free water and 0.5 µL of each primer (final concentration of 200 nM), 0.125µL of SYBR green 1 diluted in DMSO (20X) and 5µL of template DNA. The qPCR program was performed in a two-step thermal cycling procedure which consists of a pre-denaturation step for 4 min at 95°C, followed by 40 cycles of 1 min at 94.0 °C, 40 s of annealing at 56.0 °C (which was determined as the optimal annealing temperature for each primer set) and 40 s of extension at 72°C with acquisition during the extension stage. Melting curves were performed subsequently with a stepwise increase of 0.3°C from 60°C to 95°C, as a quality control. The efficiencies and R^2 values are reported in Table 3-3.

Table 3-3. qPCR efficiencies and R^2 values. *[a] The limit of quantification was determined based upon the difference between the lowest point of the standard curve and negative control reactions with additional inspection of the melting curves.*

Primer set	Efficiency	R^2	Estimated LOQ[a] (gene counts/mL)
Tim-typeII	103.63±3.89%	0.992±0.010	335
Tim-methylohigh	127.76±2.89%	0.946±0.027	2340
Arthrotim	109.87±15.03%	0.993±0.006	315
16S V3	107.81±3.87%	0.995±0.001	1360

The amount of 16S copies was recalculated to cell counts by means of a correction for the amount of 16S rRNA gene copies in the genome using the rrnDB (https://rrndb.umms.med.umich.edu/, Stoddard, Smith *et al.* (2015)) if no exact match for a certain strain was available an integer-rounded median copy number for the genus it belonged to was used. All data analyses were performed using R version 3.2.3 (http://r-project.org). The initial compatibility of a non-MOB for the MOB partner was calculated as the amount of copy-number corrected 16S rRNA gene copies of the non-MOB partner divided by the total of 16S rRNA gene copies and pmoA copies (Equation 3-1). The amount of 16S rRNA gene

copies that could be attributed to the non-MOB partner was calculated by subtracting the total 16S V3 (Table 3-2) copy number by the corresponding *pmoA* copy number (Equation 3-2).

Equation 3-1. Compatibility parameter. *$CP_{i,j}$ is the compatibility of non-MOB partner i for MOB j. $N_{i,j}$ is the copy-number corrected 16S rRNA gene count for non-MOB i co-cultivated with MOB j and M_j is the pmoA gene count for MOB j*

$$CP_{i,j} = \frac{N_{i,j}}{\left(N_{i,j} + M_j\right)}$$

Equation 3-2. non-MOB 16S rRNA gene count. *Common terms as in* ***Equation 3-1.*** *$T_{i,j}$: 16S V3 gene count of co-cultivation of non-MOB i and MOB j, R_j = pmoA/16S rRNA gene ratio for MOB j, CN_i 16S rRNA gene copy number for non-MOB i.*

$$N_{i,j} = \frac{T_{i,j} - M_j/R_j}{CN_i}$$

In Phase II of the co-cultivation experiments, the amount of variable partner was calculated by means of subtracting copy-number corrected gene counts of the *pmoA* gene (MOB) and 16S rRNA gene of the fixed partner from the total amount of 16S rRNA gene counts (Equation 3-3).

Equation 3-3. Variable partner count calculation. *$Var_{y,z}$: 16S rRNA gene count of variable partner y with MOB z. $T_{y,z}$: 16S V3 gene count of the co-cultivation. $M_{y,z}$: pmoA gene count for the co-cultivation. R_z : pmoA/16S rRNA gene ratio for MOB z. $F_{y,z}$: fixed partner 16S rRNA gene count. CN_F: 16S rRNA gene copy number for fixed partner. CN_y: 16S rRNA gene copy number for variable partner.*

$$Var_{y,z} = \frac{T_{y,z} - \frac{M_{y,z}}{R_z} - \frac{F_{y,z}}{CN_F}}{CN_y}$$

3. Results

3.1. Phase I: establishing initial 1:1 partner compatibility

A total of 38 strains comprising 19 genera and 4 taxonomic classes (Table 3-1) were co-cultivated with both an alphaproteobacterial (LMG 26262, *Methylosinus* sp.) and gammaproteobacterial (NCIMB 11130^T, *Methylomonas methanica*) MOB. By means of specific qPCR the compatibility of each of these strains with the MOB was assessed (see the Materials & Methods section). The compatibility parameter (Equation 3-1) showed a sigmoid

pattern for both MOB types (Figure 3-2 and Figure 3-3) allowing for a clear distinction of three compatibility classes: after ranking (based on the median of the biological duplicates), the first 25% of non-MOB with a greater $CP_{i,j}$ than the MOB were considered lowly compatible (VL) with the MOB, the last 25% as highly compatible (VH) and partners within an interval of 25% around the median were considered moderately compatible (VM). The compatibility did not appear to show a clear correlation with the occurrence of described methylotrophs within the genus of the non-MOB strains although overall more strains with described methylotrophy within the genus occurred within the VH class than the VL class, certainly for NCIMB 11130^T (Figure 3-2 and Figure 3-3). The relatively high value for the $CP_{i,j}$ in the control incubation of LMG 1025 without NCIMB 11130^T is due to the lacking *pmoA* detection in the denominator making the ratio close to 1 (Figure 3-2). However, the actual 16S rRNA gene count for this control was lower than any non-MOB partner and could be considered "below quantification limit", which actually caused the qPCR reaction to fail for one of the replicates (hence the horizontal bar rather than a box displayed in Figure 3-2). For LMG 26262 both replicates of this control incubation failed to amplify above the LOQ after 40 cycles of amplification (hence the lack of the resulting box in Figure 3-3). While for an axenic culture of NCIMB 11130^T $CP_{i,j}$ was 0 (as expected), it was 0.440±0.003 for LMG 26262, which could point towards contamination of LMG 26262 (not confirmed by 10% TSA plating and partial 16S rRNA gene sequencing) or background noise in the SYBR green detection. Either way, partner selection for the second phase of the experiment was only performed based on $CP_{i,j}$ greater than this value.

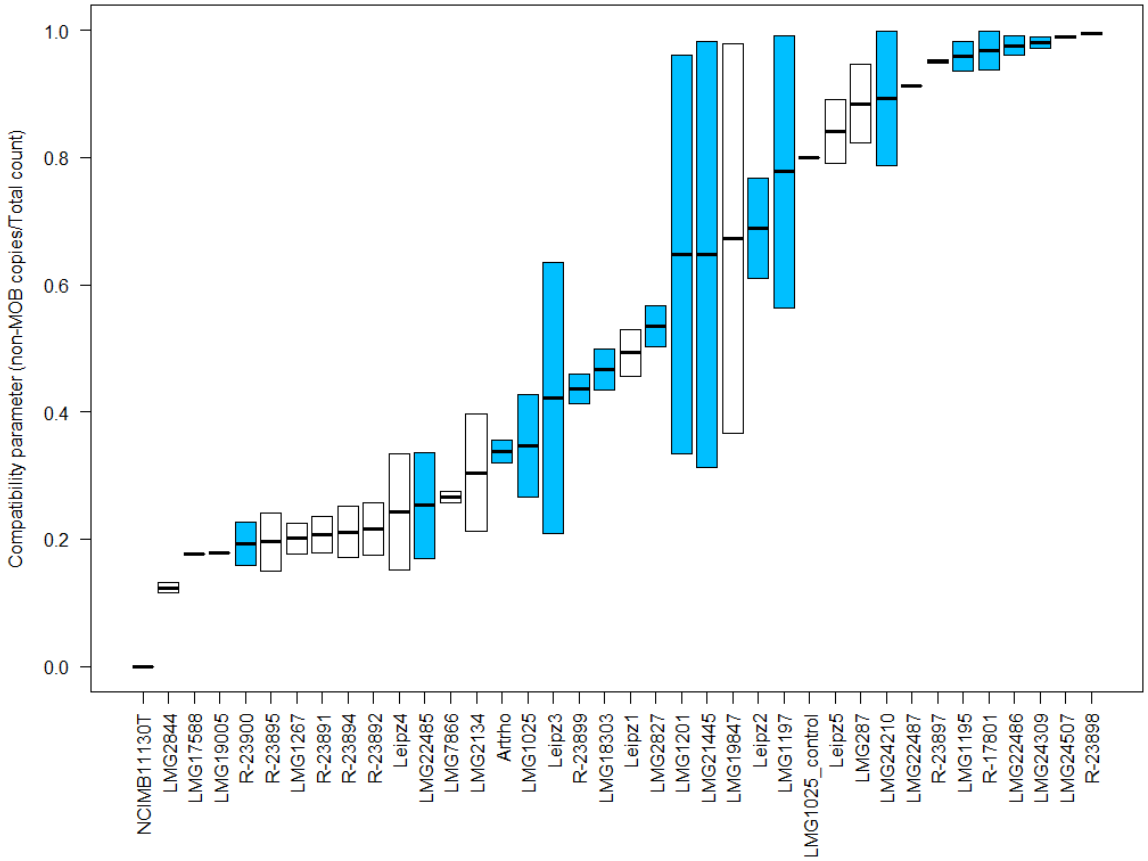


Figure 3-2. Non-MOB partner compatibility with NCIMB 11130^T. *Blue colored boxplots indicate strains with described methylotrophic members in their genus.*

To further aid in an apt selection of fixed and variable partners, apart from the compatibility parameter, the "stimulation" of MOB growth (as measured by an increase in *pmoA* gene count) was assessed (Figure 3-5). When comparing *pmoA* copy numbers it is clear that only very few strains (R-23900, R-23892, LMG 2134 and LMG 7866) could increase the *pmoA* copy number of NCIMB 11130^T whereas for LMG 26262 most non-MOB partners (except for R-23894, LMG 20219 and LMG 22487) had a positive effect on the *pmoA* gene counts as compared to axenic growth. No clear correlation could be made between described methylotrophic members of the genus and the ability of the non-MOB partners to increase *pmoA* gene counts in the 1:1 *interactome*. Given the discrepancy between the compatibility of the non-MOB and MOB growth stimulation, the absorbance of the co-culture (OD_{600}), being a proxy for total biomass, was added to the consideration for non-MOB partner selection for the second phase of the experiment (data not shown). However, a strong (Pearson product-moment correlation: 0.83) and significant correlation ($p<0.0001$ for two-sample association

test and significance of the linear regression coefficient p<0.001) between OD_{600} and *pmoA* gene counts could be observed for NCIMB 11130^T (Figure 3-4) while no such significant correlation was found for LMG 26262 (Pearson: 0.17, $P_{2\text{-sample}}$=0.32, $P_{regression}$ =0.05).

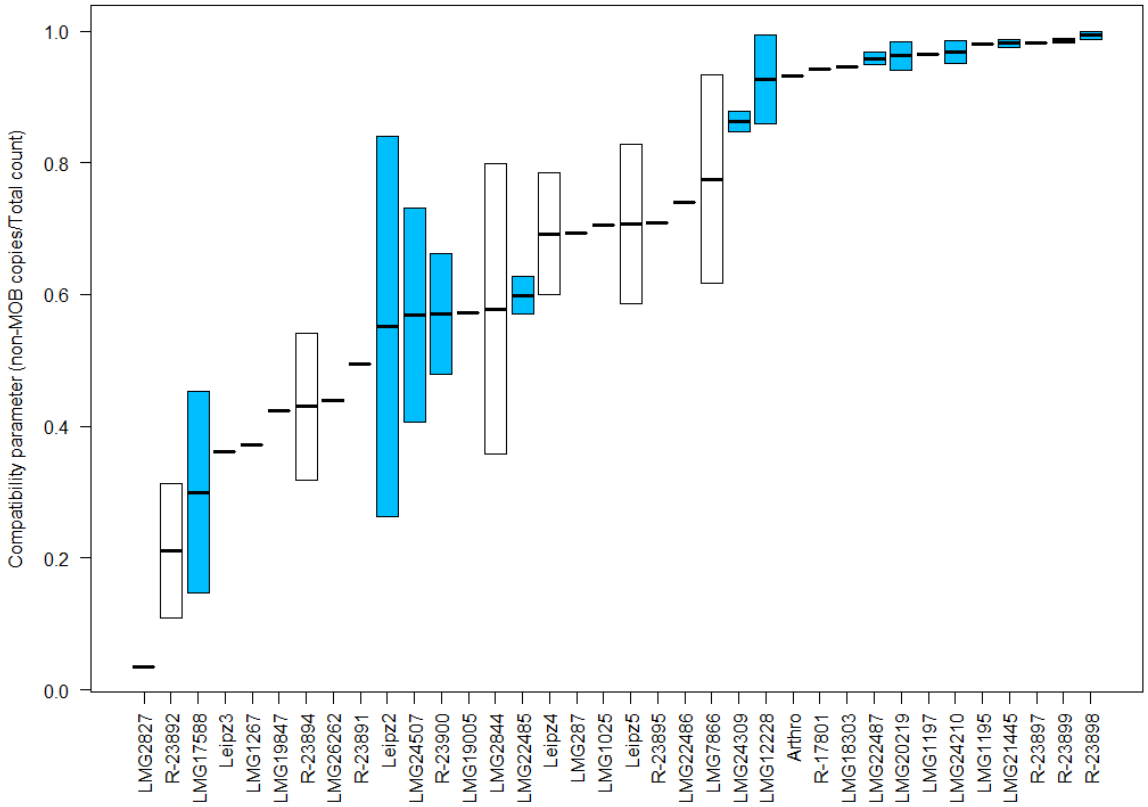


Figure 3-3. Non-MOB partner compatibility with LMG 26262. *Blue colored boxplots indicate strains with described methylotrophic members in their genus.*

Based upon the considerations outlined above, 7 strains were selected as fixed and variable partners for both MOB (Table 3-4).

Table 3-4. Selected non-MOB partners for Phase II. *The partner designation will be used in graphs and results representation for the second phase of the experiment. Optimality refers to the main criteria for choice of these specific partners.*

Non-MOB partner designation	Non-MOB partner strain (number)	Species	Optimality
FIX	Arthro	*Arthrobacter* sp.	NCIMB 11130^T VM compatibility, LMG 26262 *pmoA* count
VL1	LMG 17588	*Burkholderia multivorans*	Both MOB VL compatibility
VL2	R-23894	*Escherichia coli*	NCIMB 11130^T OD_{600} and non-MOB partner count, LMG 26262 VL compatibility
VM1	LMG 18303	*Sphingomonas*	Both MOB VM compatibility

		aromaticivorans	
VM2	LMG 7866	*Escherichia fergusonii*	NCIMB 11130^T *pmoA* count, LMG 26262 VM compatibility
VH1	R-23899	*Pseudomonas syringae* subsp. syringae	NCIMB 11130^T non-MOB partner count, LMG 26262 VH compatibility
VH2	LMG 2827	*Rhodobacter sphaeroides*	NCIMB 11130^T non-MOB partner count, LMG 26262 *pmoA* count.

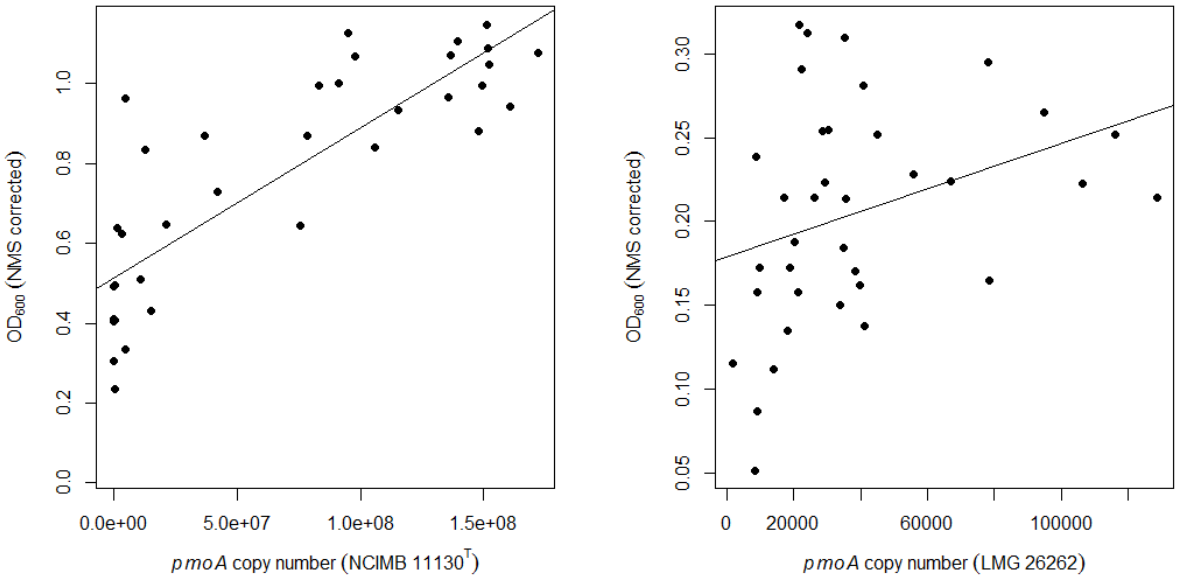


Figure 3-4. Linear model fit of OD_{600} in function of pmoA copy number for NCIMB 11130^T (left plot) and LMG 26262 (right plot). *Outliers were removed in the case of LMG 26262. Adjusted R^2 values are 0.69 and 0.01 respectively.*

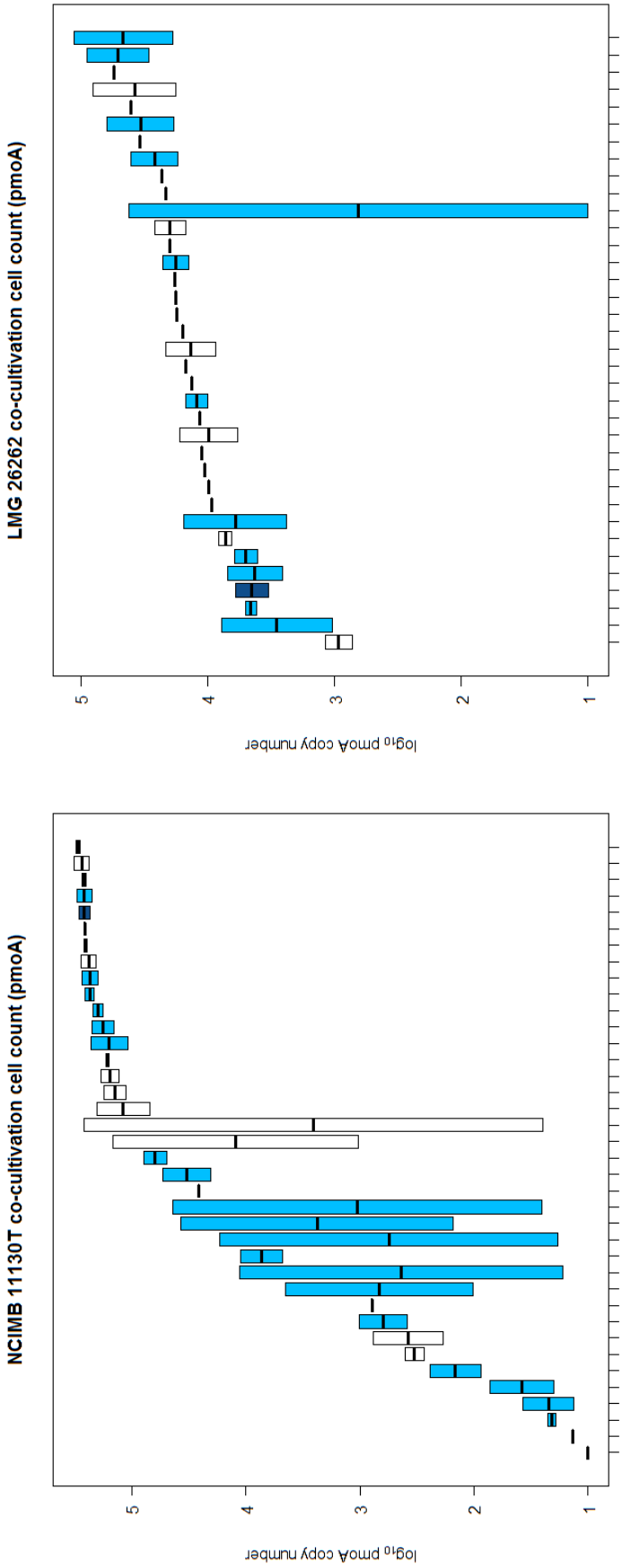


Figure 3-5. log_{10} pmoA copy numbers of the MOB in co-cultivation experiments. *Dark blue boxes indicate the MOB, light blue strains with described methylotrophs in their genus.*

3.2. Phase II: challenging the 1:1 *interactome*

Co-cultures of each MOB type (Table 3-1) with a fixed non-MOB partner (FIX, Table 3-4) were repeatedly co-cultivated for 4 cycles of 7 days (5 cycles in the case of NCIMB 11130^T). In each cycle the co-culture was challenged with each of the variable partners in triplicate (Table 3-4). We assessed how this challenge influenced functionality (CH_4 oxidation, MOR) and if the fixed partner could adapt to the MOB and persist when challenged with the variable partners. If adaptation of the fixed partner to the MOB would occur we hypothesize that it cannot be outcompeted by the variable partners. Additionally, we hypothesize that functionality (MOR) should be stimulated if the MOB and fixed partner adapt to one another.

3.2.1. Impact on functionality: methane oxidation rates

When comparing the co-cultivation MOR with the MOR of the axenic MOB overall no effect was observed for NCIMB 11130^T, with the notable exception of Cycle 1 for the MOB:FIX, MOB:FIX:VH1 and MOB:FIX:VH2 *interactomes* where the MOR was stimulated and Cycle 4 for the MOB:FIX:VM1 where the MOR was decreased (Figure 3-6).

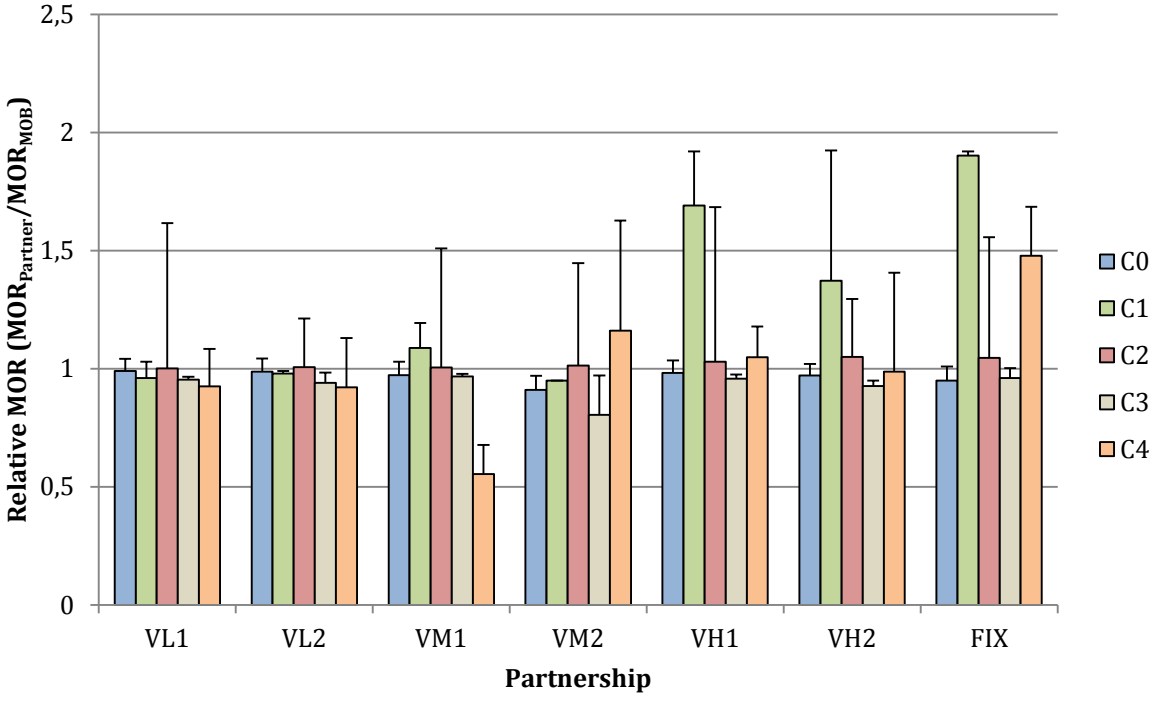


Figure 3-6. Relative MOR of co-cultivation with NCIMB 11130^T. *Coloring according to co-cultivation cycle. Error bars represent error-propagated standard deviations.*

For LMG 26262 no clear trends of the impact of co-cultivation on MOR could be observed other than the general tendency of co-cultivation to decrease MOR as compared to the axenic

culture with 10-20% regardless of co-cultivation cycle (Figure 3-7). Only for the MOB:FIX *interactome* an increase in MOR relative to the axenic MOB incubations was observed with increasing cycles of co-cultivation.

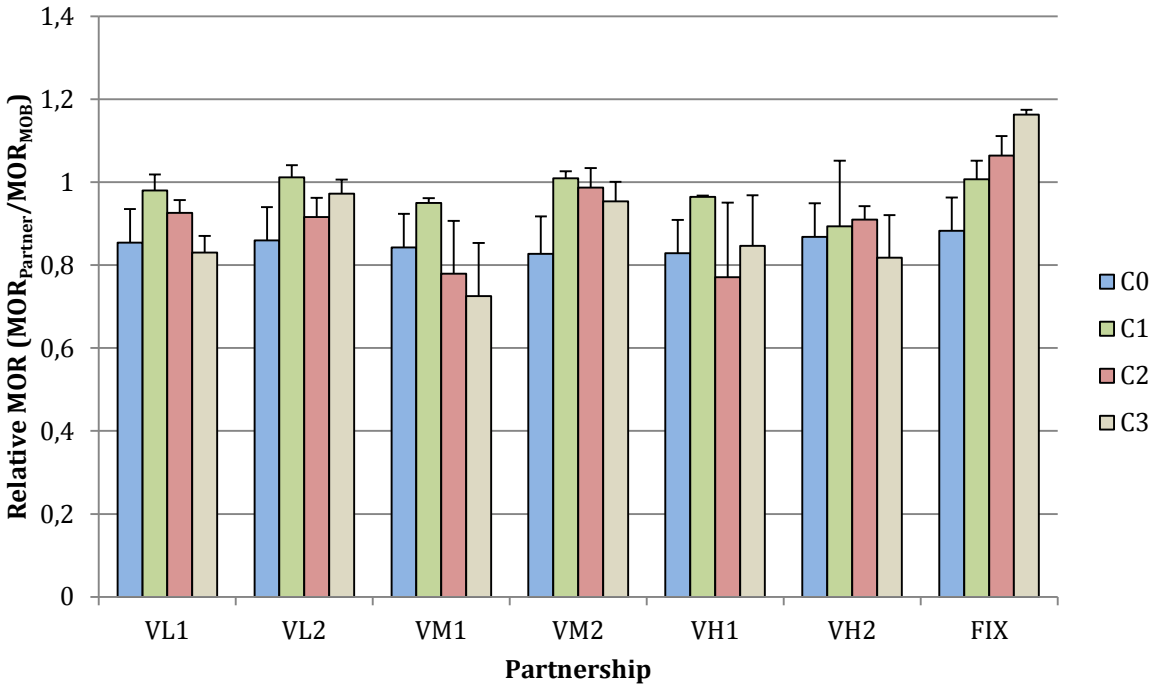


Figure 3-7. Relative MOR of co-cultivation with LMG 26262. *Coloring according to co-cultivation cycle. Error bars represent error-propagated standard deviations.*

3.2.2. Impact on the 1:1 *interactome*

The abundance of MOB, fixed partner and each variable partner were tracked through repeated cycles of co-cultivation. Overall, a reduction of fixed partner with repeated cycles of co-cultivation was observed (Figure 3-8, Figure 3-10). Although the reduction was dependent upon variable partners, no clear trends could be observed. In the case of NCIMB 11130^{T} the MOB accounted for the majority of the gene copies (Figure 3-8, Figure 3-9). The fixed partner barely contributed in abundance to the *interactome* from the first cycle of co-cultivation on. In the case of LMG 26262 the total amount of gene counts was less, though relatively more non-MOB were present and the fixed partner persisted longer in the *interactome*, dependent upon the variable partner it was challenged with (Figure 3-10, Figure 3-11).

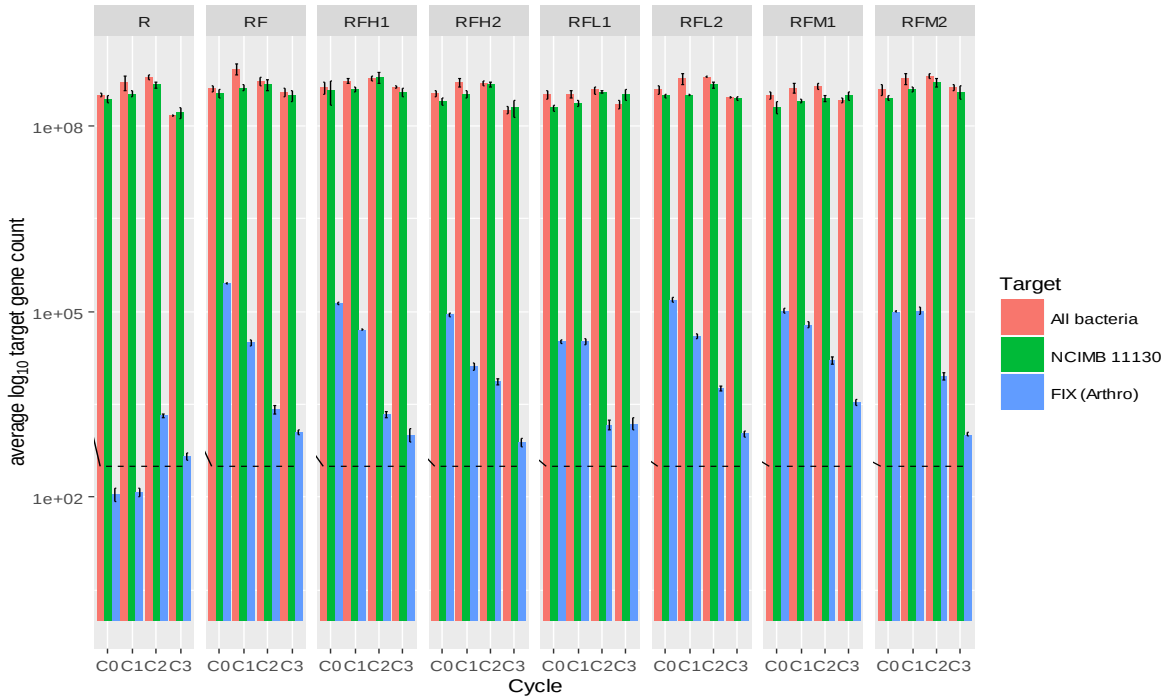


Figure 3-8. Average log_{10} scaled gene counts during co-cultivations with NCIMB 11130^T. *Targets as specified in* ***Table 3-2****. R represents axenic culture of NCIMB 11130^T while RF represents NCIMB 11130^T:FIX (Arthro) interactomes and L1,L2,M1,M2,H1 and H2 represent NCIMB 11130^T:FIX:VAR with each of the selected variable partners in* ***Table 3-4****. The dashed line indicates the limit of quantification for the Arthro primer set (****Table 3-3****).Error bars represent standard deviations.*

Figure 3-9. Average relative abundances of interactome partners with NCIMB 11130^T. *Sample groups designated as in Figure 3-8.*

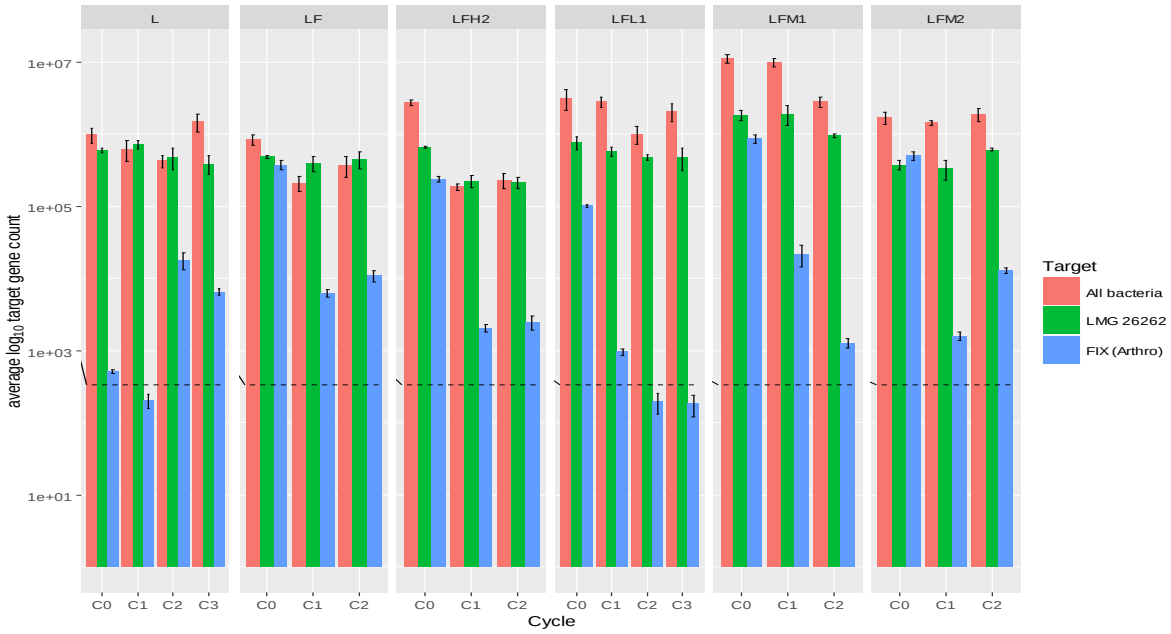


Figure 3-10. Average log_{10} scaled gene counts during co-cultivations with LMG 26262. *Targets as specified in* ***Table 3-2****. L represents axenic culture of LMG 26262 while LF represents LMG26262:FIX (Arthro) interactomes and L1,M1,M2 and H1 represent LMG26262:FIX:VAR with each of the selected variable partners in* ***Table 3-4****. The dashed line indicates the limit of quantification for the Arthro primer set (****Table 3-3****).Error bars represent standard deviations.*

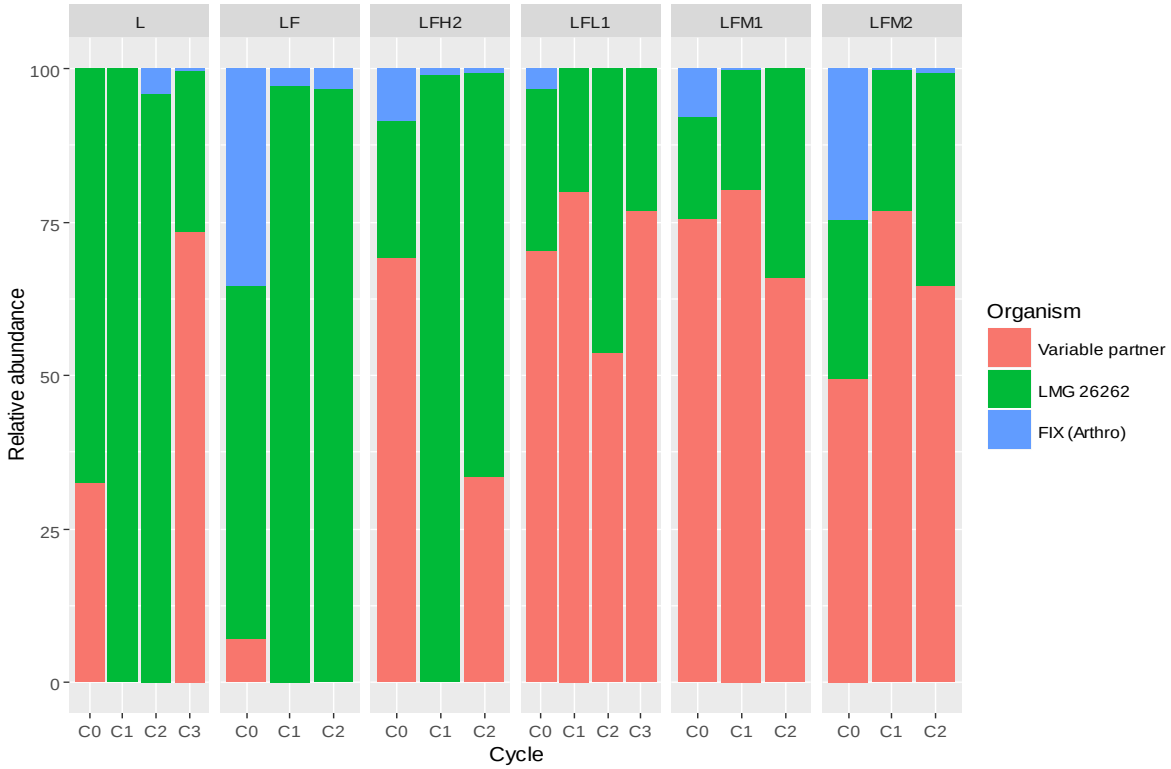


Figure 3-11. Average relative abundances of interactome partners with LMG26262. *Sample groups designated as in Figure 3-10****.***

The detected number gene counts of Arthro in the axenic incubations was always very close to the quantification limits for NCIMB 11130^T (Figure 3-8) which could be considered noise from the SYBR-green based qPCR detection and possible primer-dimer forming of the Arthro qPCR assay. On the other hand, for LMG 26262 possibly contamination may have occurred, although this was not confirmed with 10% TSA plating and partial 16S rRNA gene sequencing.

4. Discussion

4.1. Phase I: establishing compatibility in a 1:1 *interactome*

The sigmoid distribution of the compatibility parameter ($CP_{i,j}$, Figure 3-2 and Figure 3-3) indicated that indeed MOB were able to sustain different non-MOB partners to a specific extent, confirming Hypothesis 1. Given the frequent partnerships between MOB and methylotrophs, a tentative assessment was made whether the presence of a methylotrophic representative in the genus of the non-MOB strain could be linked to its compatibility with the MOB. While no clear correlation was observed, generally more strains with methylotrophs in the genus were found to occur in the high compatibility classes. However, genome mining for methylotrophy modules (Chistoserdova 2011) or experimental validation of methylotrophic metabolism of each of the individual strains is required to confirm this trend. Overall, facultative methylotrophic metabolism might be a wide-spread metabolic strategy of bacteria (Boden, Thomas *et al.* 2008; Kolb 2009; Hung, Wade *et al.* 2011; Eyice and Schäfer 2016). Although MOB were able to increase the counts of some non-MOB up to 3 (R-23899 with LMG 26262) or even 6 (Leipz3 with NCIMB 11130^T) log units, MOB *pmoA* gene counts were not stimulated to the same extent when compared to axenic MOB growth (Figure 3-5): a maximal increase of approximately 1 log unit was observed for LMG 26262 while for NCIMB 11130^T co-cultivation generally had a negative impact (up to 4 log units decrease), indicating a competitive (negative) interaction between the MOB and the non-MOB partners for the gammaproteobacterial MOB and a moderately mutualistic interaction for the alphaproteobacterial MOB. While relatively few data is available on the abundances of individual constituents in a methanotrophic co-cultivation, a 1 log unit increase (between 48 and 120 h) of *Methylovulum miyakonense* HT12 16S was observed in a co-culture with *Rhizobium* sp. Rb122 (10:1 MOB:non-MOB initial inoculation) while only a very small increase was observed in *Rhizobium* sp. Rb122 for the same time period, though no

quantification of the inoculum was performed (Iguchi, Yurimoto *et al.* 2011). Both MOB and non-MOB partners were isolated from the same methanotrophic forest soil community, which implies that the partners were co-evolved to be adapted to one another before the co-cultivation experiment. Other gammaproteobacterial MOB also exhibited stimulated growth and methane oxidation by Rb122. A co-culture of *Methylocystis* sp. M6 (Alphaproteobacteria) and *Sphingopyxis* sp. NM1 isolated from different soil consortia (hence from the same habitat but without previous adaptation), showed a density-dependent stimulation of M6 by NM1 at a 1:9 ratio of MOB:non-MOB (Jeong, Cho *et al.* 2014). The total amount of M6 co-cultivated with NM1 in a 1:9 mixing ratio was significantly higher than the control incubation (determined by FISH), additionally mRNA transcripts associated with CH_4 oxidation were upregulated. While the current experiment did not evaluate density-dependent effects, we have shown that even with a 1:1 mixing ratio growth stimulation by specific partners could be observed which confirms our previous data (Stock, Hoefman *et al.* 2013). The total biomass (measured as OD_{600}) was found to be highly correlated with the *pmoA* of gammaproteobacterial MOB whereas this was not the case for the alphaproteobacterial MOB, implying that non-MOB partners had a greater influence on co-culture OD_{600} in co-cultures with the latter MOB type. Interestingly, the two reported synthetic co-cultures above confirm the observation that in co-cultivation with alphaproteobacterial MOB a greater non-MOB partner concentration can be sustained whereas co-cultivation with gammaproteobacterial MOB results in low non-MOB to MOB ratios. This is reflected by the observation that relatively more strains had a higher compatibility ($CP_{i,j}$) with LMG 26262 (Figure 3-3) than with NCIMB 11130^T (Figure 3-2). Whether these observations can be generalised and linked with the proposed functional traits and according life strategies of alpha- and gammaproteobacterial MOB (Ho, Kerckhof *et al.* 2013) is not clear, and seminal work with continuous culture co-cultivation by Namsaraev and Zavarzin (1972) has shown abundances MOB:non-MOB ratios of 75:25 for the alphaproteobacterial MOB *Methylocystis trichosporium* 1442.

4.2. Phase II: Competition within the co-culture - no effect of adaptation

Co-cultivation with variable partners of any compatibility class did not have a clear stimulatory effect on MOR (Figure 3-6 and Figure 3-7). While this could be expected for the first cycle of co-cultivation of NCIMB 11130^T with a richness of 2 (Ho, de Roy *et al.* 2014), no adaptation effect was observed with repeated cycles of co-cultivation. Such an adaptation

effect was also not observed for a 1:1 MOB:non-MOB mixing ratio with an alphaproteobacterial MOB with repeated refreshing of the headspace CH_4/air mixture (Jeong, Cho *et al.* 2014). Notable exceptions were the stimulation of relative MOR in co-cultivation of NCIMB 11130^T with the fixed partner and variable partners VH1 and VH2 or without any variable partners for co-cultivation cycle 1, up to twice as much as the axenic culture MOR. However, the stimulatory effect on MOR did not persist during subsequent co-cultivation cycles. Additionally, while generally co-cultivation of LMG 26262 with any variable partner negatively affected MOR, a stimulation of MOR was observed with increasing co-cultivation cycles of the MOB with the fixed partner, hinting at a possible adaptation and metabolic fine-tuning of the interaction. A described increase in fitness and stability of gene-loss mutants which were obligatory cross-feeding (Pande, Merker *et al.* 2014) may be an evolutionary mechanism by which the MOB and non-MOB can adapt to one another over time. Absolute counts of the fixed partner 16S rRNA gene (Figure 3-8) could not account for the observed boost of MOR in co-cultivation cycle 1, and overall the fixed partner appeared to be "washed out" with every tenfold dilution for each cycle of co-cultivation with NCIMB 11130^T, as a reduction of approximately one log unit could be observed in the 16S rRNA counts with increasing cycle number. However, when assessing the relative count of variable partners (Figure 3-9), it could be observed that for the conditions with a boosted MOR at cycle 1 (Fix, VH1, VH2) an increased amount of variable partner 16S gene counts was present. Although this was also the case for e.g. VL2, no concomitant stimulation of MOR was observed which could be attributed to the lower compatibility of this partner with the MOB. The fact that the fixed partner could sustain itself without growing for each cycle of 7 days and only be removed by dilution may be attributed to methylovorous (rather than methylotrophic) metabolism which encompasses the dissimilatory usage of C1 compounds for energy (and maintenance) without the ability to use these compounds for biomass synthesis such as described for the ubiquitous pelagic marine heterotroph SAR 11, but believed to be wide-spread among bacteria (Sun, Steindler *et al.* 2011).

Conversely, a "wash-out" effect was not observed for every partner combination in co-cultivation with LMG 26262 (Figure 3-10). For all assessed partner combinations, the fixed partner was abundant enough to observe its relative abundance after one cycle of co-cultivation (up to 30% of the relative gene counts when no variable partner was added, Figure 3-11). Although for some variable partner incubations (VL1 and VM1) in subsequent cycles the fixed partner gene counts were severely reduced until levels around the LOQ, for VH2 and VM2 and the co-cultivation with the fixed partner alone the fixed partner was able to

persist to some extent. Although for VH2 this did not have a direct impact on the MOR, VM2 had an overall reduced negative effect on the relative MOR. Finally, the fixed partner persisted most strongly if no variable partner was added to the co-culture. Even though an initial drop of one log unit was observed, afterwards the fixed partner in co-culture with LMG 26262 sustained itself and started even recovering, which coincided with an increase in MOR.

5. Conclusion & perspectives

This research was set out to assess if preferential partnerships occur between 38 non-MOB and an alpha- and gammaproteobacterial MOB (Hypothesis 1). While a clear sigmoid profile of compatibility was observed, generally *Methylomonas methanica* NCIMB 11130^{T} (Gammaproteobacteria) sustained lower amounts of non-MOB partners than *Methylosinus* sp. LMG 26262 (Alphaproteobacteria). The non-MOB partners also rarely increased *pmoA* gene counts of NCIMB 11130^{T} while LMG 26262 *pmoA* counts could be increased up to 1 order of magnitude (i.e. tenfold). This demonstrated that preferential partnerships may be observed between MOB and non-MOB however they may not necessarily boost MOB abundance in the resulting co-culture. Next, a second hypothesis of partner adaptation (Hypothesis 2) was tested: a co-culture of an MOB and a moderately compatible partner was subjected to repeated cycles of co-cultivation. In each cycle this co-culture was challenged with variable partners with different compatibility with the MOB. Here we addressed how this adaptation would affect the functionality (MOR) and abundances of the individual partners. The adaptation hypothesis of the MOB to the fixed partner with a stimulation on MOR was only observed for a limited extent with the alphaproteobacterial MOB *Methylosinus* sp. LMG 26262.

Overall, the initial hypothesis of the adaptability of partner selection (Hypothesis 2) was largely rejected by the almost immediate wash-out of the fixed partner from the co-culture. This could be attributed to serial dilution in repeated co-cultivation batches, showing that an initial selection based upon 1 cycle of co-cultivation in Phase 1 was not appropriate. In future experiments a continuous cultivation system should be employed to ensure persistence of the partner (Namsaraev and Zavarzin 1972; Wilkinson, Topiwala *et al.* 1974; Lamb and Garver 1980). By modulating the chemostat parameters such as the dilution rate and possibly biomass retention (i.e. retentostat operation), co-cultures can be selected for specific growth rates (μ). Screening of multiple partners, as in Phase 1 of the experiment, could be performed in cheap, custom built multiplexed chemostat arrays (Miller, Befort *et al.* 2013) or micro- and

miniscale bioreactors (Lattermann and Büchs 2015), which have the additional advantage of an increased volumetric mass transfer (k_La) and maximum oxygen transfer capacity (OTR_{max}), which would be highly advantageous given the gaseous form of the CH_4/O_2 substrate for aerobic methane oxidation. Additionally, density-dependent effects of initial inoculation should be investigated as we have previously observed that low ratios of non-MOB to MOB partners could be key for establishing mutualistic rather than commensalistic interactions (unpublished data for a co-culture of gfp-labeled *P. putida* SM1699 and *Methylosinus* sp. LMG 26262). The calculation of the amount of variable partner gene counts by subtracting the MOB *pmoA* and fixed partner 16S counts (Equation 3-3) may have introduced an unwanted bias in assessing the relative distribution of non-MOB in the co-culture and a specific qPCR primer set should be developed for each of the variable partners. Alternatively, to reduce bias, a general primer set such as 16S V3 (Table 3-2) could be employed with specific qPCR probes (e.g. TaqMan) in this way a same-length amplicon resulting from the same PCR reaction is generated. Finally, to ensure identity of the partners a low-coverage NGS 16S rRNA gene amplicon sequencing run (e.g. Illumina MiSeq) could be employed to assess the relative abundance of each partner.

6. Acknowledgements & author contributions

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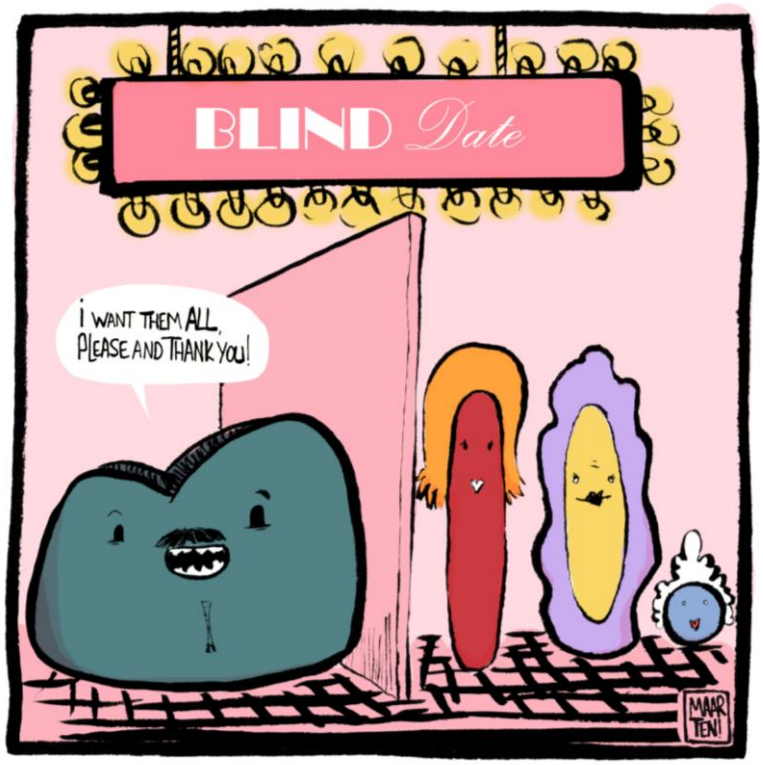
CHAPTER

4

OPTIMAL PARTNERSHIPS IN THE METHANOTROPHIC *INTERACTOME*: UNSUPERVISED SELECTION



CHAPTER 4

OPTIMAL PARTNERSHIPS IN THE METHANOTROPHIC *INTERACTOME*: UNSUPERVISED SELECTION

Abstract

Biological oxidation of methane (CH_4) is an essential ecosystem function. Accumulating evidence indicated that this function is mediated by associations of methanotrophic bacteria (MOB) with non-methanotrophic partners; together referred to as a methanotrophic *interactome*. Given the potency of CH_4 as a greenhouse gas, a thorough understanding of how these *interactomes* exert an effect on methane oxidation is of special interest. Furthermore, MOB - non-MOB associations could be exploited for sustainable biotechnological applications in light of the renewed interest in MOB as natural and cost-efficient biocatalysts. The selectivity of MOB for non-MOB partners, as well as the stimulation of MOB activity (CH_4 oxidation rate, MOR) with increasing non-MOB richness have both been recently described for a single batch incubation period. Therefore, we hypothesized that during repeated co-cultivation of MOB with non-MOB, ecological sorting would guide the methanotrophic *interactome* towards its optimal composition, which could additionally boost functionality (MOR). While no significant improvement of functionality was observed, the biological variability of MOR was stabilized by co-cultivation with non-MOB partners. Overall, higher biomass yields were obtained when MOB were co-cultivated with non-MOB partners and an alphaproteobacterial MOB appeared to be able to support more non-MOB biomass than a gammaproteobacterial MOB, which could be linked to the proposed life-strategies of these clades. A clear partner selection among non-MOB partners was observed as only 4 out of 8 initial partners were found to persist during repeated cycles of co-cultivation. While 2 of the persisting partners were found to persist with either MOB type, the other two were more restricted to a specific MOB. Differential metabolic potential of non-MOB was resolved by genome mining publicly available genomes; our attempt to find clues for the partner selectivity did not reveal a clear link with the potential for C1-compound metabolism.

However, genes for sugar metabolism (fructose, mannose, sucrose) were restricted to the persisting partners while genes encoding an ATP-dependent vitamin B12 importer were restricted to the non-persisting partners, underlining the importance of metabolic exchange in the methanotrophic *interactome*.

Chapter redrafted after:

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Definition: the (methanotrophic) *interactome*

A microbial ***interactome*** is referred here as an entire community interacting in concert to contribute to a specific microbial process. These interactions encompass (but are not limited to) (syn)trophic interactions where interacting partners exchange molecular building blocks and energy while feeding on a defined substrate. Others include antagonistic interactions such as selective predation.

1. Introduction

The biological oxidation of methane (CH_4), a greenhouse gas which contributes greatly to radiative forcing (Ciais, Sabine *et al.* 2014; Nisbet, Dlugokencky *et al.* 2014), is an essential ecosystem service which both contributes to atmospheric CH_4 removal (Kirschke, Bousquet *et al.* 2013), as well as to attenuation of terrestrial and marine methane emissions (Hinrichs and Boetius 2002; Conrad 2009). Biological methane oxidation is mainly facilitated by aerobic methane-oxidizing bacteria (MOB, methanotrophs) which possess the unique ability to use CH_4 as a sole carbon and energy source (Hanson and Hanson 1996). Due to this trait, they can act as primary producers of organic carbon in a methane-driven ecosystem (Petersen and Dubilier 2009; Ruff, Arnds *et al.* 2013; van Duinen, Vermonden *et al.* 2013). Accumulating evidence shows that MOB are generally present in so-called methanotrophic *interactomes,* consisting of MOB and non-MOB partners, and these *interactomes* as a whole are likely the main contributors to enhanced biological methane oxidation (Stock, Hoefman *et al.* 2013; Ho, de Roy *et al.* 2014; Iguchi, Yurimoto *et al.* 2015; Oshkin, Beck *et al.* 2015). Given the importance of CH_4 as the third most potent greenhouse gas (Yusuf, Noor *et al.* 2012), an in-depth understanding of the underlying interactions in biological methane removal is of great interest. Furthermore, apart from cycling of CH_4, interactions within the methanotrophic *interactome* or its interactions with other non-methanotrophic *interactomes* may influence biogeochemical cycling of other building blocks of life on earth (e.g. N or S) (Costa, Perez *et al.* 2006; Luesken, Sanchez *et al.* 2011; Joye 2012; Hanke, Hamann *et al.* 2014; Ho, de Roy *et al.* 2014; Kraft, Tegetmeyer *et al.* 2014; Koch, Lucker *et al.* 2015; Oshkin, Beck *et al.* 2015). This is evidenced, for instance, by the reports on the interactions between MOB and ammonia-oxidizing Archaea and Bacteria (AOA/AOB) (Daebeler, Bodelier *et al.* 2014; Zheng, Huang *et al.* 2014) and could possibly be reflected by the differentiation in nitrogen metabolism within the *Methylococcaceae*/*Methylophilaceae* interaction (Hernandez, Beck *et al.* 2015). Additionally, the importance of accompanying bacteria for MOB-driven biotechnological processes has been illustrated in the case of single-cell protein production (SCP) (Bothe, Jensen *et al.* 2002), CH_4-driven denitrification (Amaral, Archambault *et al.* 1995; Modin, Fukushi *et al.* 2007; Zhu, Wang *et al.* 2016) and the methane-driven production of biopolymers (Helm, Wendlandt *et al.* 2006).

In natural systems, MOB are known to interact with higher organisms (Petersen and Dubilier 2009; van der Ha, Bundervoet *et al.* 2011; Bao, Okubo *et al.* 2014; Putkinen, Larmola *et al.* 2014; Iguchi, Yurimoto *et al.* 2015) however, we limit our discussion of the methanotrophic

interactome here to associations between MOB and bacterial non-MOB partners (Hanson and Hanson 1996; Hrsak and Begonja 2000; Iguchi, Yurimoto *et al.* 2011; Stock, Hoefman *et al.* 2013; Jeong, Cho *et al.* 2014). While the benefit of associating with the MOB is clear for non-MOB, i.e. acquisition of organic carbon from CH_4 through MOB (Murase and Frenzel 2007; Modin, Fukushi *et al.* 2010), it is less straightforward what benefit the MOB acquire from their non-MOB partners (Iguchi, Yurimoto *et al.* 2015). One way in which non-MOB partners aid MOB is the removal of inhibitory intermediates from CH_4 metabolism, such as methanol, as evidenced by the frequent association of methylotrophic non-MOB with MOB (He, Wooller *et al.* 2012; Beck, Kalyuzhnaya *et al.* 2013; Takeuchi, Kamagata *et al.* 2014; Danilova, Suzina *et al.* 2016). Additionally, toxic intermediates of MOB-driven degradation of organic (micro)pollutants (Benner, De Smet *et al.* 2015) can be detoxified by associated non-MOB partners (Hrsak and Begonja 2000; Hesselsoe, Boysen *et al.* 2005). Alternatively, non-MOB can supply growth factors (such as vitamins) to the MOB, as evidenced by rhizobial strains which stimulated gammaproteobacterial growth and CH_4 oxidation by excreting cobalamin (vitamin B12) (Iguchi, Yurimoto *et al.* 2011). Although some experiments have shown that partnerships between the MOB and non-MOB partners are highly specific (Stock, Hoefman *et al.* 2013; van der Ha, Vanwonterghem *et al.* 2013; Hernandez, Beck *et al.* 2015), an increase in community diversity (richness, evenness), rather than specific partnerships, influenced ecosystems functionality (Wittebolle, Marzorati *et al.* 2009) within the methanotrophic *interactome* (Ho, de Roy *et al.* 2014).

The detailed study of these complex microbial interactions is hampered by technical limitations. Even by means of an established top-down method such as stable isotope probing (SIP) (Dumont and Murrell 2005; Neufeld, Wagner *et al.* 2007) causal evidence for cooperation and/or evidence for a bidirectional flux between partners in natural ecosystems is hard to establish. Hence recently, synthetic ecology experiments have been considered as a bottom-up approach in the toolkit of the environmental microbiologist (Jousset, Schulz *et al.* 2011; Mee and Wang 2012; De Roy, Marzorati *et al.* 2014; Faith, Ahern *et al.* 2014; Großkopf and Soyer 2014; Bai, Müller *et al.* 2015; Stenuit and Agathos 2015). Although (re)assembling an ecosystem by means of isolates is not a new concept (Namsaraev and Zavarzin 1972; Wilkinson, Topiwala *et al.* 1974), the recent rise in interest for these type of experiments illustrates the need for a greater understanding as to how biotic interactions influence biogeochemical cycling.

In this study, the hypothesis that MOB select for specific non-MOB partners based on their ability to stimulate the functionality of the MOB (i.e. methane oxidation rate; MOR) was

tested by adding a temporal variable to the co-cultivation (i.e. multiple cycles of repeated co-cultivation) of the methanotrophic *interactome* as recently employed by Ho, de Roy *et al.* (2014) in the context of the richness-functionality relationship. Additionally, both a representative alphaproteobacterial and gammaproteobacterial MOB were included, as these clades harbor the majority of the currently described and cultivated MOB (Semrau, DiSpirito *et al.* 2010; Knief 2015), and both clades are hypothesized to have distinct life strategies (Ho, Kerckhof *et al.* 2013). Although these life strategies (e.g. competitiveness) could potentially impact biotic interactions with non-MOB in a methane-driven ecosystem, it is not yet known if and how they influence the methanotrophic *interactome*. If MOB show preference for specific partners, prolonged co-cultivation will select for these preferred partners. Conversely, the incompatible non-MOB partners will recede with successive cycles of co-cultivation. Hence, in this experiment, repeated co-cultivation will result in an unsupervised self-selection of the most optimal methanotrophic *interactome*. In support, the difference in the genetic make-up of the preferred partners was mined for cues on the potential mechanism of selectivity. Additionally, the influence of these preferred partnerships on the stability of ecosystems functionality (i.e. variability in MOR) was determined.

2. Material and methods

2.1. Strains and growth conditions

Methylomonas methanica NCIMB 11130^T (Gammaproteobacteria; type I) and *Methylosinus* sp. LMG 26262 (Alphaproteobacteria, type II) were chosen as methanotrophs. Non-methanotrophic partner cultures were selected and cultivated as described previously based upon comparable growth conditions and stimulatory or neutral interaction with the MOB (Ho, de Roy *et al.* 2014) (Table 4-1). After resuscitation from a -80 °C stock, the bacteria were inoculated on nutrient agar (Sigma-Aldrich, 70148) for 14 h at 28° C, after which they were transferred to liquid LB broth (Lennox, Sigma L3022) and placed on an orbital shaker (120 rpm) at 28° C for 30 h. MOB (triplicate) and non-MOB partner co-cultures (quadruplicate), as well as a partner-only control (unreplicated) were grown on 20 mL NMS medium (with copper) (Whittenbury, Phillips *et al.* 1970) in 120 mL opaque serum bottles closed air-tight with grey butyl rubber stoppers. The bottles were incubated on a rotary shaker (150 rpm) at 28 °C. At the start of the batch incubation, the headspace of the serum bottles contained 20% (v/v) CH_4 (N45, Air Liquide, Belgium) in air. The synthetic communities were assembled at

equal starting numbers for each strain based on cell count using flow cytometry (Van Nevel, Koetzsch *et al.* 2013). The initial MOB-non-MOB co-culture inoculum contained 10^8 cells mL^{-1}. Both axenic cultures of MOB and partners contained the same number of cells as was present in the synthetic ecosystem per organism type. Serial co-cultivation was performed by transferring 10% (v/v) to fresh NMS medium after approximately 72 hours per cycle. An overview of the experimental design and sampling amounts is given in Figure 4-1.

Table 4-1. Primary consumer strains used in the co-cultivation experiment (Ho, de Roy et al. 2014). *([a]) "yes" (+publication): restricted facultative (obligate) or facultative methylotrophy is described within the species; "yes for strain": genomic evidence shows the possibility of methylotrophy (Chistoserdova 2011), within confirmed methylotrophic species; "unclear": contradictory information was found in the literature, the genome, or both; "no": no evidence for methylotrophy was found in literature or in the genome of the strains in the table.*

Strain	Strain number	Methylotrophic ([a])	Genome source
Paracoccus denitrificans	LMG 4049	Yes for strain	IMG/ER: tax ID 2597490357
Rhizobium radiobacter	LMG 287	No	GOLD project: Gp0000707
Ochrobactrum anthropi	LMG 2134	No	GOLD project: Gp0000090
Cupriavidus metalluridans	LMG 1195^T	No	GOLD project: Gp0000357
Comamonas terrigena	LMG 1249	No	GOLD project: Gp0023602
Achromobacter denitrificans	LMG 1231^T	unclear	GOLD project: Gp0033444
Pseudomonas putida	LMG 24210	No	GOLD project: Gp0000136
Escherichia coli	LMG 2092^T	No	GOLD project: Gp0110161

2.2. Analytical methods

Headspace gas composition was sampled concurrently at 0, 4, 6, 24, 48 and 72 hours during cycle 1, 3, 4 and 5. The headspace was sampled and injected in a Compact GC® (Global Analyser Solutions, the Netherlands) equipped with a PoraBOND Q pre-column (Agilent, USA), a Molsieve 5A column, one channel connected to a flame ionization detector and two channels connected to a thermal conductivity detector. The system was controlled by EZChrom Elite software (Agilent, USA).

Total cellular protein was determined using the BioRad DC Protein Assay, which is a modified version of the Lowry protein quantification as per the manufacturer's instructions. Lyophilized bovine serum albumin was used as a protein standard. Previously, MOB growth was shown to have a linear correlation with total protein measurements (Hoefman, van der Ha *et al.* 2014).

Total heterotrophic plate counts were performed at the end of the fifth co-cultivation cycle by decimal serial dilution plating (using sterile physiological solution) on nutrient agar (Sigma-Aldrich, 70148). Plates were counted after 48h of incubation at 28°C. Only plates with more than 30 or less than 300 cells were counted, and counts were assumed to be Poisson distributed.

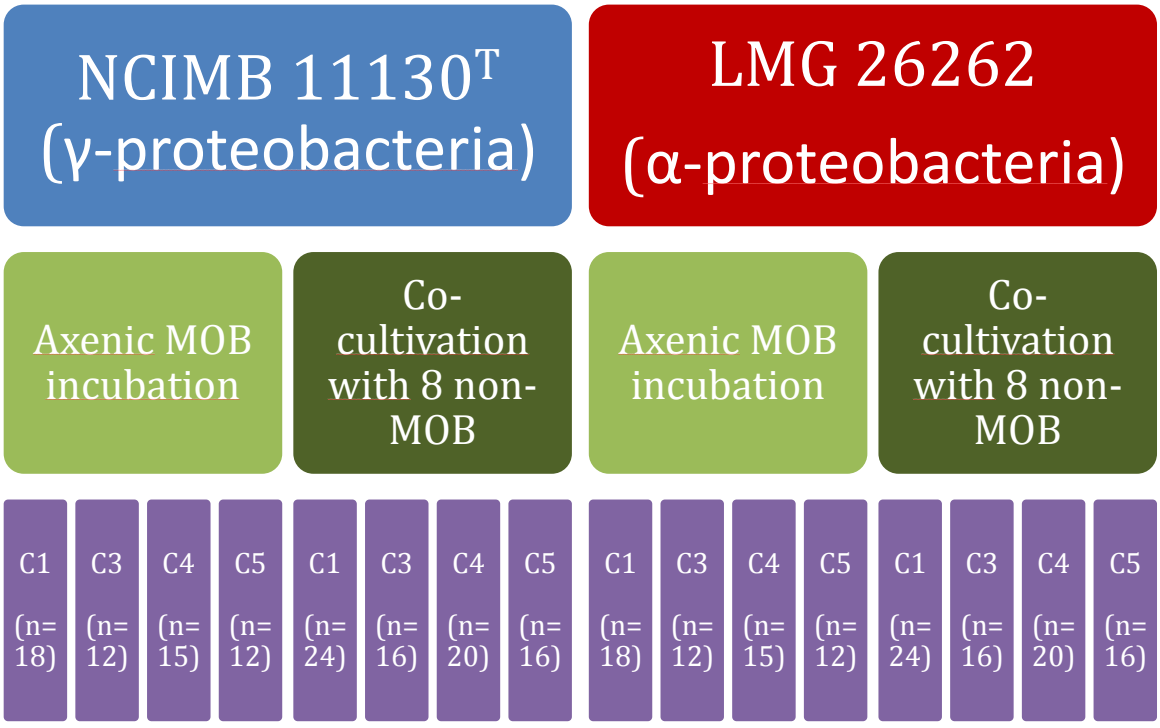


Figure 4-1. Experimental design. *C(1-5) represents the cycle of (co)-cultivation, where n represents the total amount of headspace measurements available for each cycle. Colors are added for interpretability.*

2.3. PCR-DGGE

A 16S rRNA gene region was amplified by PCR using 338F and 518R primers targeting the V3 region (Muyzer, Dewaal *et al.* 1993; Ovreas, Forney *et al.* 1997). A GC clamp of 40 bp (Muyzer, Dewaal *et al.* 1993; Ovreas, Forney *et al.* 1997) was added to the forward primer. The PCR program consisted of 10 min 95°C; 35 cycles of 1 min. 94°C, 1 min. of 53°C, 2 min. of 72°C; and a final elongation for 10 min. at 72°C. Amplification products were analysed by electrophoresis in 1.5% (wt/vol) agarose gels stained with ethidium bromide. DGGE (Denaturing Gradient Gel Electrophoresis) based on the protocol of Muyzer, Dewaal *et al.* (1993) was performed using the INGENYphorU System (Ingeny International BV, The Netherlands). PCR fragments were loaded onto 8% (w/v) polyacrylamide gels in 1 × TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA pH 7.4). To process and compare the different gels, a homemade marker of different PCR fragments was loaded on each gel (Boon, De Windt *et al.* 2002).The polyacrylamide gels were made with denaturing gradients ranging from 40% to 60% (where 100% denaturant contains 7 M urea and 40% formamide). The electrophoresis was run for 16 hours at 60°C and 120V. Staining and analysis of the gels was performed as described previously (Boon, Goris *et al.* 2000). The normalization and analysis of DGGE gel patterns was done with the BioNumerics software 5.10 (Applied Maths, Sint-Martens-Latem, Belgium), which was also used to assign band classes. Distinctive band classes for each *interactome* partner were selected based upon patterns from individual axenic cultures that were loaded on the DGGE.

2.4. Statistical data analysis

All statistical analyses and data visualizations were performed using R 3.2.3 (http://r-project.org). Multiple comparisons were performed as follows: after checking normality of residuals (both by means of a Q-Q normal plot as well as the Shapiro-Wilks normality test) and homogeneity of variances (both by means of inspecting boxplots as well as robust Levene-type testing or Brown-Forsythe testing if normality could not be assumed) the following general and post hoc tests were performed: if the normality and homoscedasticity hypothesis could be retained an ANOVA was performed with Tukey HSD Post-Hoc testing. If normality was retained but homoscedasticity rejected a weighted-least squares ANOVA was run with a Games-Howell post-hoc test. If normality could not be retained nonparametric multiple contrast effects were employed with Tukey contrasts. To model the methane removal generalized additive models were employed in R. Variance-to-mean ratios (VMR) were used

as relative dispersion metrics and were calculated by dividing the cycle means and variances of normalized methane oxidation rates.

2.5. Comparative genomics

Publically available genomes were acquired from their respective sources (Table 4-1) and annotated by the rapid annotations using subsystems technology (RAST) server using default settings ("classic RAST" annotation scheme, RAST as a gene caller, FIGfam release 70, Genetic code 11, automatically fix errors and backfill gaps) with additional fixing of frame shifts and construction of a metabolic model (Aziz, Bartels *et al.* 2008; Overbeek, Olson *et al.* 2014). After annotation, a genbank file was downloaded from RAST and pathway-genome databases were built using the PathoLogic tool in PathwayTools (Karp, Paley *et al.* 2010) v. 19.5 with automatic build and all automated options in the consistency checker. Subsequently, comparative analyses were run using the web interface of the PathwayTools web server.

3. Results and discussion

3.1. MOB partner selection

The specificity of MOB interaction with non-MOB partners in the co-cultures (Chapter 3, section 3.1) could lead to the selection of partners by the MOB after repeated co-cultivation. Indeed, previous reports on 1:1 co-cultivation experiments of 25 non-MOB with 9 MOB partners showed that specific combinations of MOB and non-MOB differentially stimulated maximal growth and growth rates of the resulting 1:1 *interactomes* during a single co-cultivation cycle (Stock, Hoefman *et al.* 2013). Furthermore, the sigmoid partner compatibility curves in Chapter 3 (Figure 3-2 and Figure 3-3) confirm that differing MOB/non-MOB ratios can occur within the *interactome* which strengthens the hypothesis of partner specificity. In the current experiment, 8 partners were combined at equal concentrations of $3.17\pm1.75*10^7$ cells mL^{-1} to the incubation with $3.23\pm2.31*10^7$ cells mL^{-1} of either *Methylomonas methanica* NCIMB 11130^T or *Methylosinus* sp. LMG 26262. By adding an initial excess of non-MOB as compared to MOB only the non-MOB best adapted for interaction with the MOB will be able to persist in the *interactome* after repeated cycles of co-cultivation. Hence, this unsupervised selection for specific non-MOB partners in the methanotrophic *interactome* will be mediated by competition among the non-MOB partners for specific CH_4-derived carbon sources supplied by the MOB. Alternatively, the negative and

neutral interactions observed by Stock, Hoefman *et al.* (2013) could indicate partner incompatibility with MOB-derived carbon, leading to immediate washout upon repeated sub-cultivation. The presence/absence (and relative abundance) of each individual constituent partner of the methanotrophic co-culture was determined at the end of each co-cultivation cycle using denaturing gradient gel electrophoresis (DGGE). As expected, the MOB were observed in each cycle and each treatment condition (Figure 4-2 and Figure 4-3). Additionally, of the eight initial non-MOB partners, only the same four strains (*R. radiobacter* LMG 287, *C. metallidurans* LMG 1195^T, *A. denitrificans* LMG 1231^T, *P. putida* LMG 24210) could be observed at the end of the first co-cultivation cycle with each of the MOB. These partners could hence be considered persisting partners. *R. radiobacter* LMG 287 and *C. metallidurans* LMG 1195^T were present in all cycles for both MOB and were therefore viewed as "promiscuous" partners (Figure 4-2). Co-cultivation was more selective towards *A. denitrificans* LMG 1231^T and *P. putida* LMG 24210, which were more specific partners for *Methylosinus* sp. LMG 26262 and *M. methanica* NCIMB 11130^T, respectively (Figure 4-2). Of the partners which could not be detected in the first cycle (the non-persisting partners) only *P. denitrificans* LMG 4049 re-appeared in cycle 5 of co-cultivation with LMG 26262. This suggests that during previous cycles its biomass did not wash out but rather remained present at levels below DGGE detection limits. We have previously observed this behavior when *Methylosinus* sp. LMG 26262 was combined with a GFP-tagged *Pseudomonas putida* (strain SM1699 obtained from prof. Søren Molin; Sternberg, Christensen *et al.* (1999)): low initial inoculum concentrations and a 5/95 non-MOB/MOB ratio were found to be required for optimal adaptation and development of a mutualistic interaction (F.M. Kerckhof & Charlotte De Rudder, unpublished data). To our knowledge, this is the first report of a repeated co-cultivation experiment which tracks the presence/absence of both MOB and non-MOB partners and additionally shows specific partners selection depending upon the MOB type for a defined set of non-MOB partners.

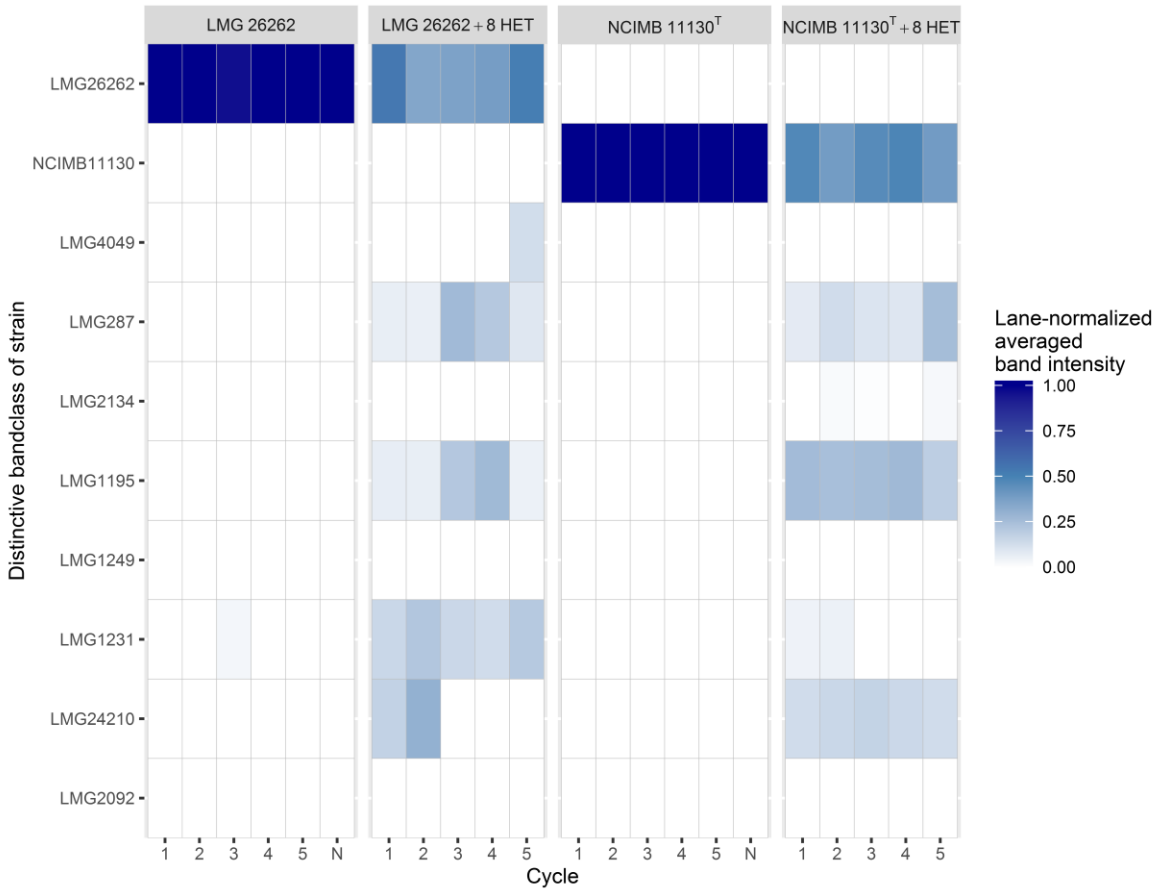
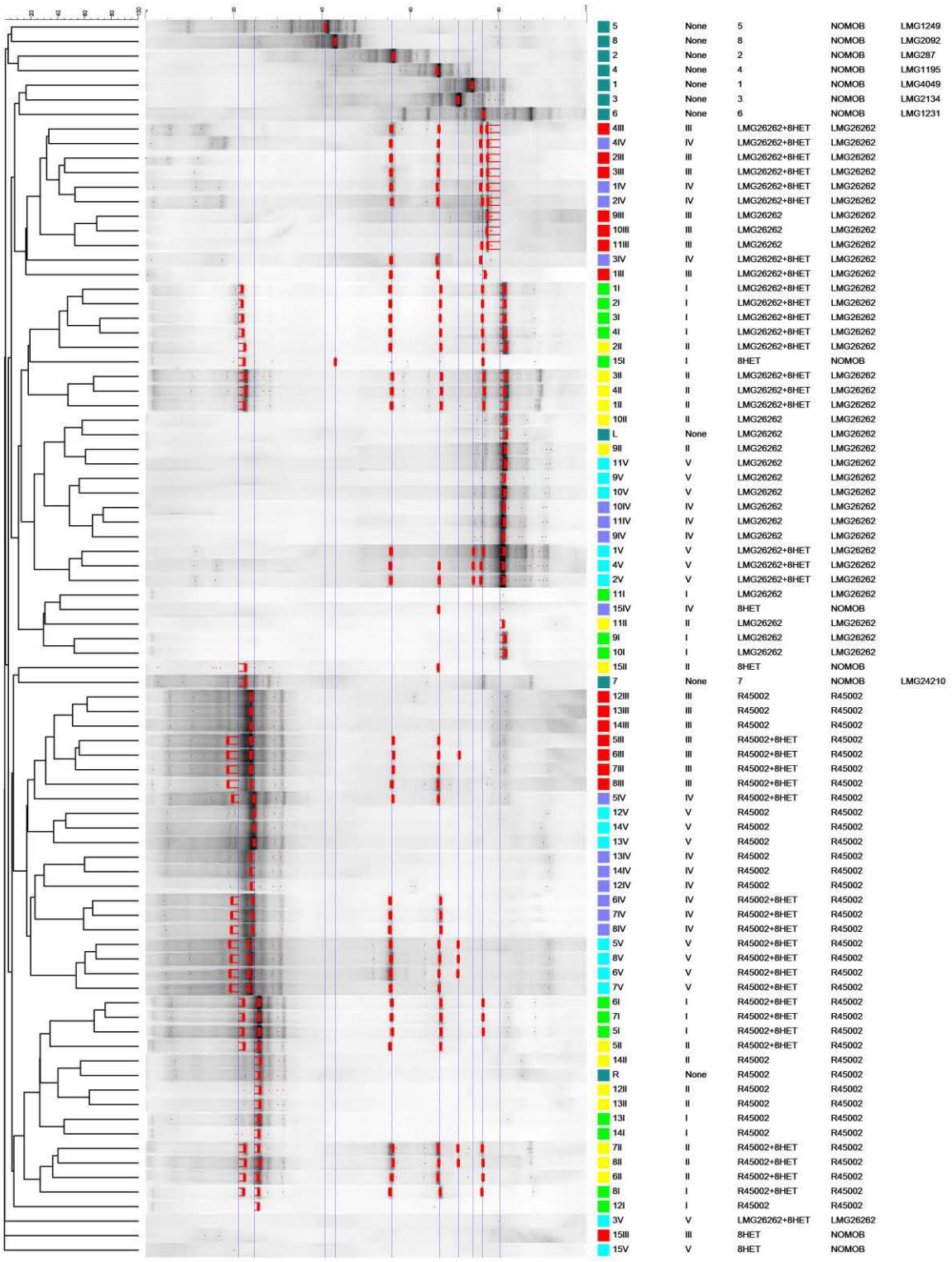


Figure 4-2. Community structure heatmap by 16S rRNA DGGE. *Representative bandclasses were assigned to the MOB and each non-MOB partner (*Figure 4-3*). Each cell represents the average relative band intensity over all biological replicates (n=3 for pure MOB and n=4 with non-MOB partners, n=1 if cycle="N"). The color intensity of the cells is proportional to the relative abundance. Cycles 1 through 5 are indicated with their corresponding number, and "N" indicates an axenic culture was loaded in the lane. "+8HET" indicates co-cultivation incubations with 8 initial non-MOB partners (as described in the materials & methods section).*

Figure 4-3. DGGE pattern among the cycles (underlying data for Figure 4-2). *16S rRNA gene DGGE was performed as described in materials & methods. Using BioNumerics (Applied Maths, version 5.1) band classes were assigned. Only the most abundant band class of a pure strain loaded on the gel was selected as "representative" band class, hence correcting for possible ghost bands. Samples were color-coded according to the cycle they belonged to. R45002 represents Methylomonas methanica NCIMB 11130^T. +8HET refers to co-cultivation incubations. 8HET as such are lanes from the negative control of the 8 non-MOB incubated without the MOB. Cycle "none" refers to pure cultures loaded in the lane. Fuzzy clustering was performed using the Jaccard distance (aware of band intensity) and UPGMA linkage.-* ***Figure is displayed on the next page.***


338 518 DGGE ingeny
338 518 DGGE ingeny
5 None 5 NOMOB LMG1249
8 None 8 NOMOB LMG2092
2 None 2 NOMOB LMG287
4 None 4 NOMOB LMG1195
1 None 1 NOMOB LMG4049
3 None 3 NOMOB LMG2134
6 None 6 NOMOB LMG1231
4III III LMG26262+8HET LMG26262
4IV IV LMG26262+8HET LMG26262
2III III LMG26262+8HET LMG26262
3III III LMG26262+8HET LMG26262
1IV IV LMG26262+8HET LMG26262
2IV IV LMG26262+8HET LMG26262
9III III LMG26262 LMG26262
10III III LMG26262 LMG26262
11III III LMG26262 LMG26262
3IV IV LMG26262+8HET LMG26262
1III III LMG26262+8HET LMG26262
1I I LMG26262+8HET LMG26262
2I I LMG26262+8HET LMG26262
3I I LMG26262+8HET LMG26262
4I I LMG26262+8HET LMG26262
2II II LMG26262+8HET LMG26262
15I I 8HET NOMOB
3II II LMG26262+8HET LMG26262
4II II LMG26262+8HET LMG26262
1II II LMG26262+8HET LMG26262
10II II LMG26262 LMG26262
L None LMG26262 LMG26262
9II II LMG26262 LMG26262
11V V LMG26262 LMG26262
9V V LMG26262 LMG26262
10V V LMG26262 LMG26262
10IV IV LMG26262 LMG26262
11IV IV LMG26262 LMG26262
9IV IV LMG26262 LMG26262
1V V LMG26262+8HET LMG26262
4V V LMG26262+8HET LMG26262
2V V LMG26262+8HET LMG26262
11I I LMG26262 LMG26262
15IV IV 8HET NOMOB
11II II LMG26262 LMG26262
9I I LMG26262 LMG26262
10I I LMG26262 LMG26262
15II II 8HET NOMOB
7 None 7 NOMOB LMG24210
12III III R45002 R45002
13III III R45002 R45002
14III III R45002 R45002
5III III R45002+8HET R45002
6III III R45002+8HET R45002
7III III R45002+8HET R45002
8III III R45002+8HET R45002
5IV IV R45002+8HET R45002
12V V R45002 R45002
14V V R45002 R45002
13V V R45002 R45002
13IV IV R45002 R45002
14IV IV R45002 R45002
12IV IV R45002 R45002
6IV IV R45002+8HET R45002
7IV IV R45002+8HET R45002
8IV IV R45002+8HET R45002
5V V R45002+8HET R45002
8V V R45002+8HET R45002
6V V R45002+8HET R45002
7V V R45002+8HET R45002
6I I R45002+8HET R45002
7I I R45002+8HET R45002
5I I R45002+8HET R45002
5II II R45002+8HET R45002
14II II R45002 R45002
R None R45002 R45002
12II II R45002 R45002
13II II R45002 R45002
13I I R45002 R45002
14I I R45002 R45002
7II II R45002+8HET R45002
8II II R45002+8HET R45002
6II II R45002+8HET R45002
8I I R45002+8HET R45002
12I I R45002 R45002
3V V LMG26262+8HET LMG26262
15III III 8HET NOMOB
15V V 8HET NOMOB

3.2. From CH_4 to *interactome* biomass: CH_4 oxidation and CH_4-C distribution

To investigate the significance of the observed association between the two MOB and the eight different non-MOB partners belonging to 3 classes and 8 genera, CH_4-oxidizing activity and the fate of CH_4-derived carbon were determined during the repeated co-cultivation experiments. As a control, the non-MOB partners were incubated under the same conditions without MOB. Unexpectedly, no significant differences in methane oxidation rates (MOR, mmol CH_4 oxidized L^{-1} h^{-1}) were observed between axenic MOB and MOB with partners ($p>0.05$) in cycle 1 and subsequent cycles of repeated co-cultivation. Additionally, fitting of generalized additive (mixed) models did not show a significant effect of non-MOB partners on the methane removal profiles (Figure 4-4) at the 5% significance level (data not shown). However, the type of MOB always had a significant effect ($p<0.05$, data not shown) which is also clear from the overall higher average MOR for *M. methanica* NCIMB 11130^T (0.60±0.33 mmol CH_4 oxidized L^{-1} h^{-1}, averaged over all cycles) as compared to *Methylosinus* sp. LMG 26262 (0.24±0.25 mmol CH_4 oxidized L^{-1} h^{-1}) , reflecting the different traits, and possibly, life strategies adopted by the MOB (Ho, Kerckhof *et al.* (2013)): gammaproteobacterial MOB were classified as competitors/competitors-ruderals in a competitor-stress tolerator-ruderals (C-S-R) framework, derived from ecological theory for plants (Grime 1977). This implies that gammaproteobacterial MOB are considered to be highly competitive for nutrients, trace elements and CH_4 and oxygen (C), while simultaneously being highly robust to disturbances (such as grazing, heat/cold stress, desiccation/rewetting (Ho, van den Brink *et al.* 2016): C-R). Conversely, alphaproteobacterial MOB are believed to be stress tolerators/stress tolerators-ruderals which implies that they thrive with increasing stress (such as low levels of CH_4, O_2, nutrients but also physicochemical stress: S).

In contrast to previous findings (Ho, de Roy *et al.* 2014) this experiment showed no significant activity differences between incubation of *M. methanica* NCIMB 11130^T with or without non-MOB partners (which was also observed for *Methylosinus* sp. LMG 26262, specific to this experiment). This could be attributed to differences in the experimental setup, e.g. a subset of the partners of the previous work was used and added these partners were added in higher initial amounts than before. Nevertheless, the results showed that addition of non-MOB partners reduced the overall variability of the MOR, hence "stabilizing" the biological variability of methane removal by both axenic MOB strains, regardless of the co-cultivation cycle. The 'stabilizing' effect appears to become stronger with repeated sub-cultivation as compared to Cycle 1 (Figure 4-5, Figure 4-6). This stabilization of MOR in the

presence of non-MOB partners may be attributed to the removal of inhibitory compounds (Wilkinson, Topiwala *et al.* 1974; Hanson and Hanson 1996), which corroborates with earlier findings that community diversity enhances functional stability (Wittebolle, Marzorati *et al.* 2009). A possible mechanism behind this stabilization could be phenotypic heterogeneity: Auto-inhibitory compounds of CH_4-metabolism by the MOB could lead to a stochastically divergent response in pure MOB cultures: even among biological replicates the resistance of axenic MOB to these compounds could be dependent upon mutations or regulations that occur stochastically (as has been demonstrated for ethanol resistance of E. coli (Swings, Wuyts *et al.* 2015) or switching to methanol metabolism by *Methylobacterium extorquens* (Strovas and Lidstrom 2009) and phenotypic diversity of *Bacillus subtilis* under carbon starvation (de Jong, Veening *et al.* 2012)), leading to phenotypic (physiological) heterogeneity in isogenic microbial populations (Davidson and Surette 2008; Lidstrom and Konopka 2010; Reinhard and van der Meer 2013; Ackermann 2015). Addition of non-MOB partners could relieve the stress (challenge) of these inhibitory compounds. Hence, by increasing the diversity of the methanotrophic *interactome* (by adding non-MOB partners) different inhibitory compounds could be removed from the culture. Process stabilization of the production of single cell protein (SCP, BioProtein) by *Methylococcus capsulatus* Bath was shown for an *interactome* with *Ralstonia* and Bacillales (Bothe, Jensen *et al.* 2002). These non-MOB, which were initially considered "contaminants" of pure culture SCP production by *M. capsulatus*, were afterwards deliberately introduced to the *M. capsulatus* culture to enhance growth and process stability. Predictable and reliable functionality (here represented by methane removal and biomass growth) are essential for biotechnological application of synthetic ecosystems (Pandhal and Noirel 2014).

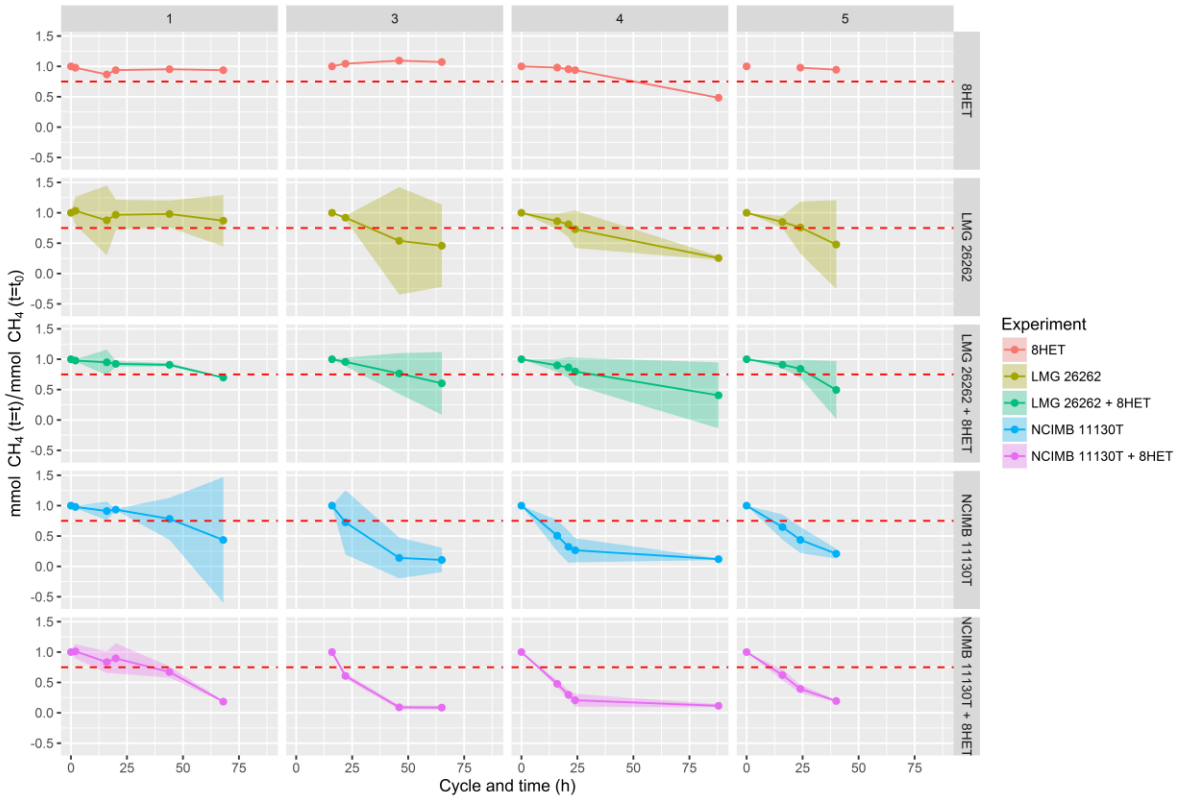


Figure 4-4. Methane removal profiles. *C/C_0 profiles for all experimental conditions and cycles. Observation points represent average C/C_0 of either triplicate (MOB alone) or quadruplicate (MOB with heterotrophs) measurements, except for the heterotroph control incubation without methanotrophs, where only one biological replicate was used. A dashed horizontal red line represents 25% methane removal from the initial concentration. Shaded areas represent 95% confidence intervals on the observations. Colors of lines and data points represent the experimental groups: in red the non-MOB negative control incubations (8HET) are shown, in brown/green the incubations with only LMG 26262 without non-MOB partners are shown, in dark green the co-cultivation incubations of LMG26262 with the non-MOB (+ 8HET) are shown in blue the incubations of only NCIMB 11130^T without non-MOB partners are shown and in magenta the co-cultivation incubations of NCIMB 11130^T with the non-MOB (+ 8HET) are shown.*

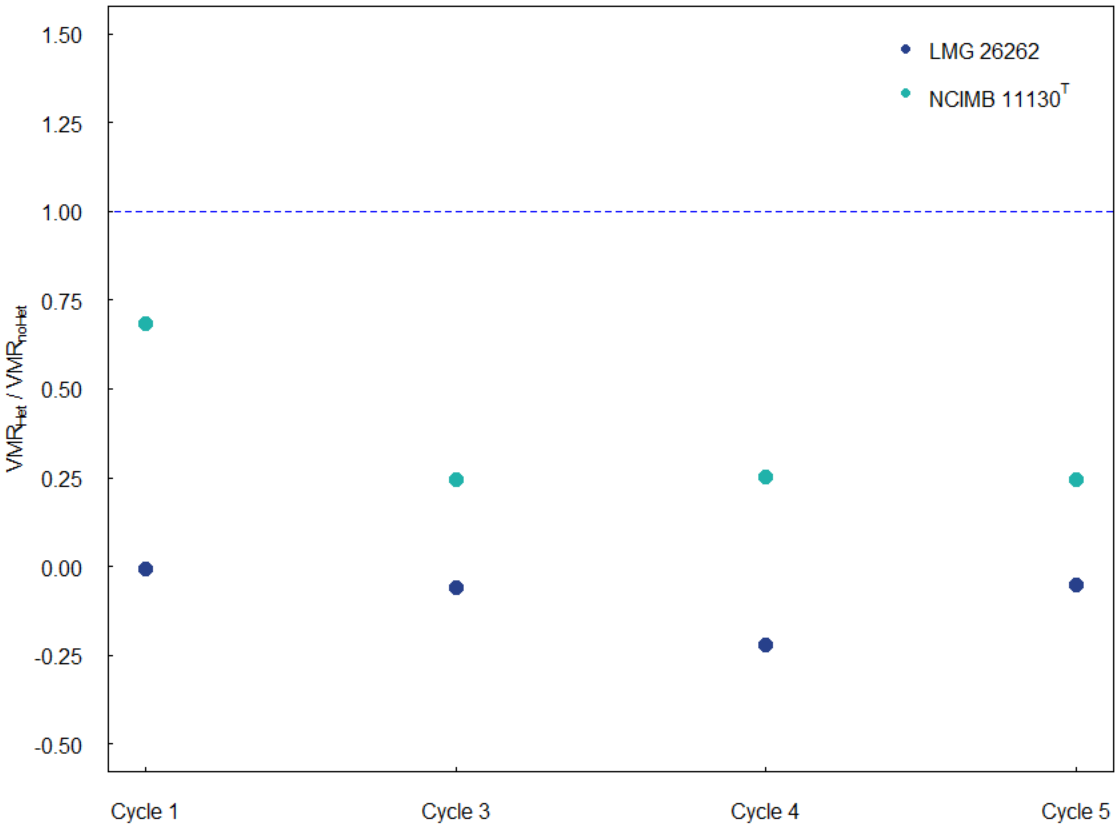


Figure 4-5. Stabilization of methane oxidation rates (MOR) by co-cultivation. *The stabilization of MOR is visualized by the ratio of Variance-to-Mean ratios (VMR) of the MOR from the co-cultivation of Methylosinus sp. LMG 26262 or M. methanica NCIMB 11130^T with eight heterotrophic partners over the MOR from the axenic MOB incubations. A dashed line represents a ratio of 1 in which case the relative MOR variance with partners is as high as without partners. Values below the dashed line indicate a lower VMR when the MOB is accompanied by the partners.*

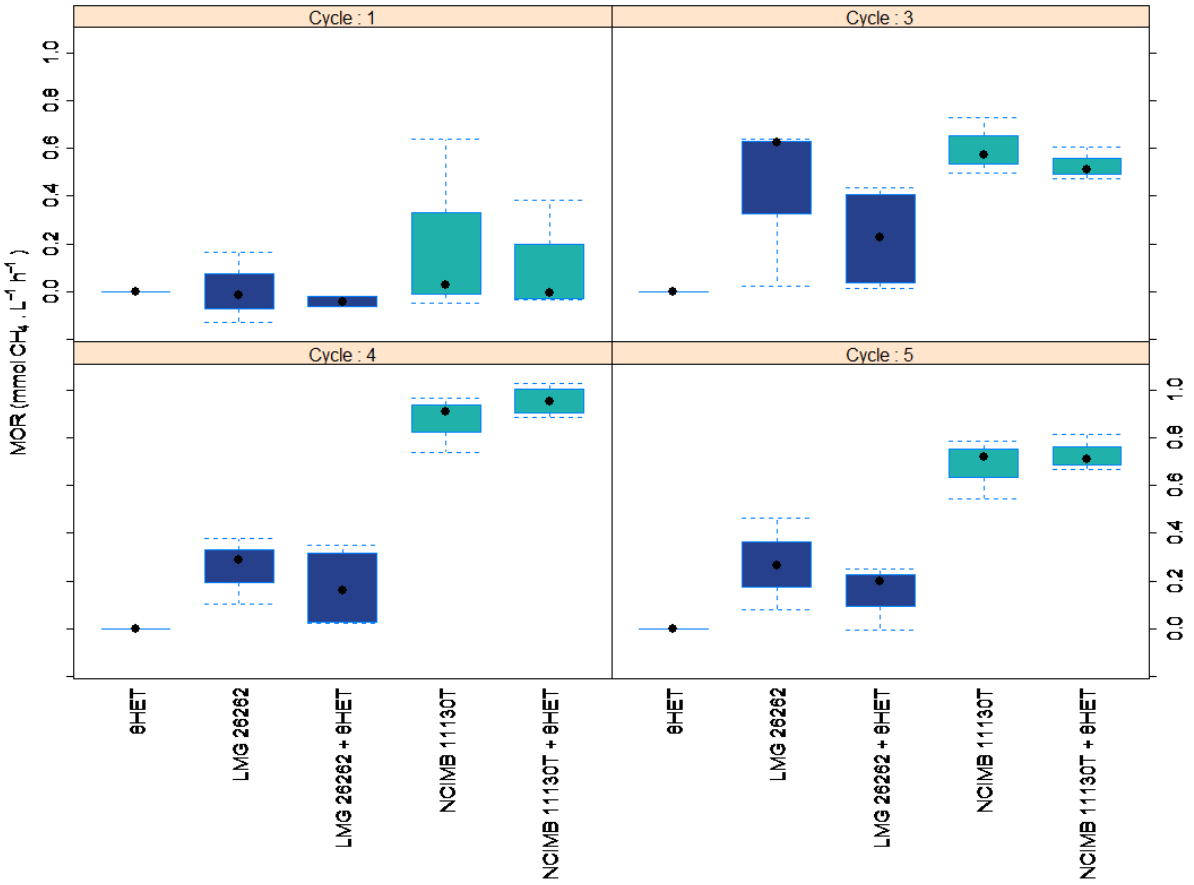


Figure 4-6. Box-and-whisker plots for methane oxidation ratio's (MOR) for each cycle per treatment. *MOR is expressed as mmol CH_4 oxidized per liter of broth per hour. MOR is corrected for losses during incubation by means of a negative control with only heterotrophs (HET). LMG: pure culture cultivation of Methylosinus sp. LMG 26262. LMG+HET: co-cultivation of Methylosinus sp. LMG26262 with 8 non-MOB partners as described in materials & methods. R: pure culture cultivation of Methylomonas methanica NCIMB 11130^T. R+HET: co-cultivation of Methylomonas methanica NCIMB 11130^T with 8 non-MOB partners as described in materials & methods. Black dots in the boxplot represent the median MOR.*

To track how CH_4 derived carbon was distributed towards CO_2 (catabolism) and biomass (anabolism), the total cellular protein as well as the headspace CO_2 were measured through all cycles. Overall, more CH_4-C was converted to biomass, and thus less to CO_2, when MOB were incubated with partners (Figure 4-6). This suggests a higher yield of total protein produced per amount of CH_4 consumed when partners were co-cultivated with the MOB (Table 4-2) and, to a lesser extent, to a decrease of CO_2 produced per amount of CH_4 consumed (Table 4-2). More protein per mg of CH_4 was synthesized during co-cultivation with *M. methanica* NCIMB 11130^T than with *Methylosinus* sp. LMG 26262. Interestingly,

total heterotrophic plate counts were lower for NCIMB 111130^T ($1.30*10^8 \pm 1.14*10^4$ CFU mL^{-1}) than LMG 26262 ($1.68*10^9 \pm 4.10*10^4$ CFU mL^{-1}), suggesting that the anabolic stimulation of partners might be MOB-type specific and could be coupled to their proposed life-strategies. For instance, the competitor *M. methanica* NCIMB 11130^T likely assimilated more of the CH_4-C than the stress-tolerator *Methylosinus* sp. LMG 26262, leaving less C available to the non-MOB partners. Here too, we confirmed this observation by an experiment in which both MOB were combined in same amounts with a GFP-tagged (Sternberg, Christensen *et al.* 1999) *Pseudomonas putida* SM1699, which resulted in a higher GFP event count (determined by flow cytometry) for co-cultivation with *Methylosinus* sp. LMG 26262 than with *M. methanica* NCIMB 11130^T after 6 repeated cycles of 72 h (F.M. Kerckhof & C. De Rudder, unpublished data). An additional differentiating feature between both MOB was the increased CO_2 production during each cycle for *Methylosinus* sp. LMG 26262, which could not be observed for *M. methanica* NCIMB 11130^T. When partners were added, the ratio of CO_2-C to protein was generally lower for each respective cycle in the case of *Methylosinus* sp. LMG 26262 (Figure 4-7), mainly due to an increase in the amount of total protein (biomass) synthesized. This effect could not be observed for *M. methanica* NCIMB 11130^T. Negative controls with axenic non-MOB partners confirmed absence of growth and lack of CO_2 production indicating carbon was primarily derived from CH_4 in the presence of MOB. Moreover, protein amounts were below the detection limit in all cycles and time points which suggest a lack of growth without the MOB and CH_4 (data not shown). Consequently, the increased amount of biomass-protein synthesized without an increase in MOR (in the case of co-cultivation with *Methylosinus* sp. LMG 26262) indicates that non-MOB growth was sustained by more efficient CH_4-C turnover: as compared to axenic MOB incubations, the additional assimilation into protein could only be attributed to the non-MOB partners. This would require metabolic fine-tuning of MOB and non-MOB partners to optimally distribute and assimilate the CH_4-C. Furthermore, the additional aerobic respiration by the non-MOB partners could benefit growth of *Methylosinus sp.* LMG 26262 as alphaproteobacterial MOB rely on the serine cycle for the assimilation of CH_4 derived carbon (Equation 1-1 and Figure 1-7), which requires 1 mol of CO_2 per 2 mol of assimilated formaldehyde. Hence, the consumption of the additional CO_2 from non-MOB respiration may have boosted total biomass increase as compared to axenic MOB incubations. Increased levels of CO_2 have been shown to stimulate biomass increase in a soil methanotrophic *interactome*, driven by an alphaproteobacterial MOB (Acha, Alba *et al.* 2002). Overall, reports on co-cultivation influencing MOR (Ho, de Roy *et al.* 2014; Jeong, Cho *et al.* 2014) are more scarce than

reports on increased growth (biomass yield) of the methanotrophic *interactome* as compared to a pure culture (Wilkinson, Topiwala *et al.* 1974; Hrsak and Begonja 2000; Bothe, Jensen *et al.* 2002; Stock, Hoefman *et al.* 2013; Jeong, Cho *et al.* 2014) , which could indicate that although MOR and biomass increase are highly linearly correlated for axenic MOB cultures (Hoefman, van der Ha *et al.* 2014), a decoupling of these processes may occur within the methanotrophic *interactome*. Hence the use of an *interactome* rather than axenic MOB may not be advantageous if CH_4 removal is the desired application (e.g. CH_4 biofiltration), though it may be advantageous in cases where overall biomass production is the goal (e.g. SCP or PHB production from CH_4).

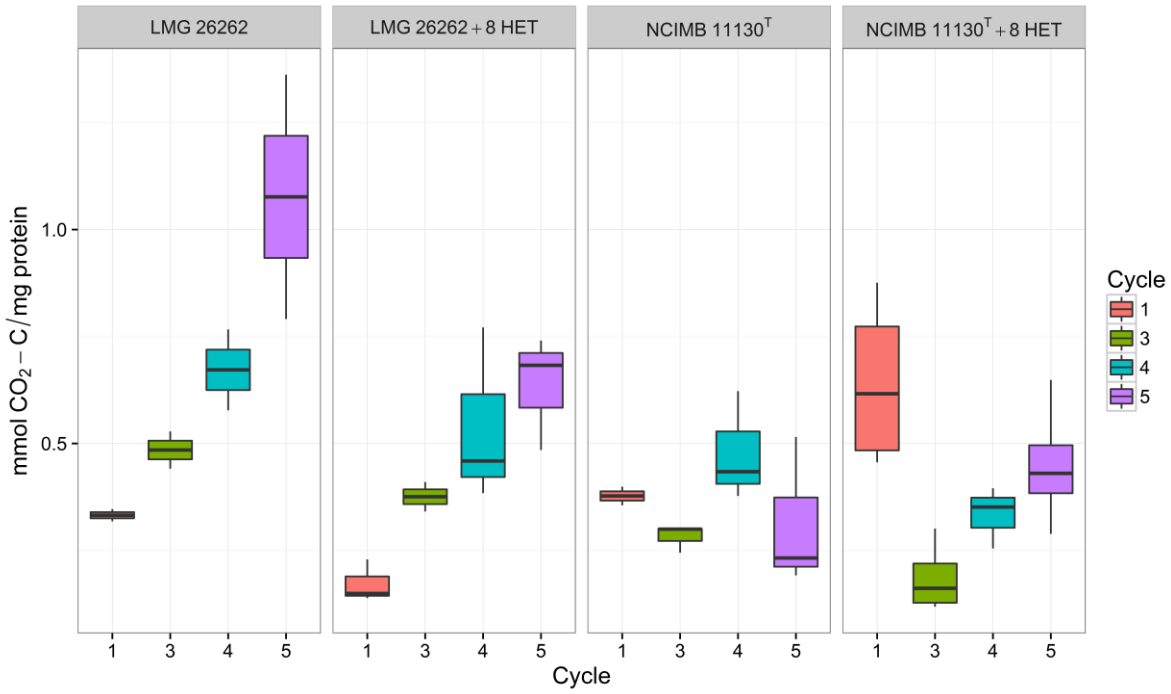


Figure 4-7. Box-and-whisker plots of carbon ratio of CO_2-C formed per mL over mg total protein per mL at the end of each cycle. *No headspace or protein measurements were performed for cycle 2. The heading of each group of boxplots describes the treatment. Strain numbers of the MOB alone represent axenic culture conditions. "+8HET" designates co-culture with 8 non-MOB partners (as described in the materials & methods section).*

Table 4-2. Yield coefficients. *Δ designates increase or removal between t_0 and t_{end} of a cycle, where t_0 for the amount of protein was calculated based upon the 10% v/v transfer at the end of cycle 3.*

Yield (mg/mg)	Cycle	*M. methanica* NCIMB 11130^T	*M. methanica* NCIMB 1130^T + 8 HET	*Methylosinus* sp. LMG 26262	*Methylosinus* sp. LMG 26262 + 8 HET
ΔCO_2/ Δprotein	4	1.240±0.352	0.882±0.230	1.677±0.339	1.521±0.807
	5	0.490±0.073	1.142±0.457	2.849±1.114	1.645±0.429
Δprotein/ ΔCH_4	4	0.620±0.144	0.751±0.100	0.607±0.139	0.739±0.407
	5	1.641±0.181	0.837±0.315	0.379±0.111	0.647±0.156
ΔCO_2-C/ ΔCH_4-C	4	0.272±0.050	0.257±0.066	0.362±0.010	0.354±0.009
	5	0.296±0.012	0.311±0.006	0.371±0.038	0.372±0.011

3.3. On the mechanism of partner selection: clues from genome mining

Although the underlying mechanisms governing the specificity of non-MOB partner selection remain inconclusive (Hernandez, Beck *et al.* 2015), some clues on the ecological significance of the observed partner selectivity were found through comparative genomics. This was greatly facilitated by the synthetic ecology approach in this work which allowed use of strains with full genome availability.

A thorough comparison of genes encoding for different methylotrophy modules (compiled from Chistoserdova (2011) and RAST scenario's/subsystems, see also Chapter 1, section 2.2.1) showed limited distinctive genes in any module investigated. In primary oxidation modules pyrroloquinoline quinone (PQQ) synthase, a cofactor of (methanol) dehydrogenases, was found to be primarily restricted to persisting partners (except for *R. radiobacter* LMG 287). No corresponding genes for methanol oxidation were found in any non-MOB partner except *P. denitrificans* LMG 4049. The promiscuous persisting partners exclusively encoded for presumed oxidation modules for methylated sulfur species (methylsulfonates, dimethylsulfide and dimethylsulfoniopropionate). Finally, both *P. denitrificans* LMG 4049 and *A. denitrificans* LMG 1231^T were the only non-MOB encoding for a primary oxidation module for methylamine. Furthermore, no further discrimination between persisting and non-

persisting partners could be made based on genes for C1-metabolism. However, other differences in carbon metabolism could be observed: D-threo-aldose 1-dehydrogenase (E.C. 1.1.1.122), an enzyme involved in fructose and mannose metabolism was only encoded by persisting partners. Additionally, a gene encoding glucoamylase (E.C. 3.2.1.3), involved in starch and sucrose metabolism was restricted to persisting partners. These genes could be involved in metabolism of sugars derived from soluble EPS synthesized by the MOB (Wilshusen, Hettiaratchi *et al.* 2004; van der Ha 2013; Wei, Su *et al.* 2015); extremophile MOB have even been reported to directly produce sucrose (Medvedkova, Khmelenina *et al.* 2007; But, Khmelenina *et al.* 2015; Khmelenina, Rozova *et al.* 2015). Several genes involved in β-alanine biosynthesis and degradation were also found to be restricted to persisting partners.

Considering transporters, nearly all non-persisting partners (except *O. anthropi* LMG 2134) encoded for an ATP-dependent cobalamin importer (Vitamin B12 ABC transporter, ATPase component BtuD) in their genome while the persisting partners were all lacking this gene. This may indicate that partners able to compete with the MOB for cobalamin were selected against persistence within the methanotrophic *interactome*. Interestingly, only the genome of *M. methanicia* NCIMB 11130^T was found to encode for this functionality, while it was not observed in *Methylosinus* sp. LMG 26262. Cobalamin (synthesized by rhizobia) has previously been shown to stimulate gammaproteobacterial methanotrophs (Iguchi, Yurimoto *et al.* 2011), although there was only a weak effect for *Methylomonas methanica* S1. A gene encoding cobalamin synthase (E.C. 2.7.8.26) was encoded by all non-MOB partners (except for *A. denitrificans* LMG 1231^T), but not in the MOB. Regardless of the gene inventory, measurement of cobalamin synthesis under the current growth conditions should be performed to assess gene expression (and cobalamin export). However, the differential presence of a cobalamin transporter in persisting versus non-persisting partners adds to the increasing evidence that cobalamin is of importance for biological methane oxidation (Lamb and Garver 1980; Iguchi, Yurimoto *et al.* 2011; Hoefman, van der Ha *et al.* 2014; Iguchi, Yurimoto *et al.* 2015) and its role requires further in-depth investigation.

4. Conclusions and perspectives

In this experiment, it was shown that co-cultivation of non-MOB partners reduced the variability in MOR that was observed with axenically-grown MOB. Furthermore, the co-cultivation with the alphaproteobacterial methanotroph *Methylosinus* sp. LMG 26262 had a

higher biomass yield (measured as total protein) as compared to a pure culture, however this effect was not observed for the gammaproteobacterial MOB *Methylomonas methanica* NCIMB 11130^{T}. Of the 8 non-MOB partners that were initially added to both MOB, only 4 could be detected after the first sub-cultivation, showing selectivity of the MOB towards their non-MOB partners. The underlying mechanisms for partner selection still need to be resolved. Therefore, we mined the genomes of the non-MOB for clues on possible driving mechanisms of MOB- non-MOB interactions. Indeed, the availability of the full genome of each constituent strain (as in this experiment) is advantageous for other 'omics' applications to further unravel how gene-expression (metatranscriptomics) and translation to proteins (metaproteomics) is influenced by repeated co-cultivation and possible adaptation of MOB and their partners in a methanotrophic *interactome*. Additionally, since all carbon in the *interactome* is derived from CH_4, ^{13}C-labeled SIP approaches coupled to meta-proteomics and metabolomics can directly link these observed adaptations to CH_4 oxidation and assimilation. Finally, investigations into the spatiotemporal structure (and the exchanged metabolites, by e.g. nano-desorption electrospray ionization (Watrous, Roach *et al.* 2012)) of interactions could allow for proper modeling and hence manipulation of the methanotrophic *interactome* (Larsen, Hamada *et al.* 2012; Jagmann and Philipp 2014; Widder, Allen *et al.* 2016). Ultimately, this modeling could supply the tools for adequate microbial resource management (Verstraete, Wittelbolle *et al.* 2007; Read, Marzorati *et al.* 2011) for increased *recovery* of the carbon and energy harnessed in CH_4 rather than merely removing CH_4 to CO_2 (Verstraete 2015).

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FMK & CDR designed and performed the experiments, analyzed the data and wrote the manuscript. RP performed statistical data analysis and performed a cricital review of the manuscript. AH, KH and NB designed the experiment and performed a critical revision of the manuscript.

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CHAPTER

5

PRESERVING THE (METHANOTROPHIC) *INTERACTOME*

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Abstract

The use of mixed microbial communities (microbiomes) for biotechnological applications has steadily increased over the past decades. However, these microbiomes are not readily available from public culture collections, hampering their potential for widespread use. The main reason for this lack of availability is the lack of an effective cryopreservation protocol. Due to this critical need, the functionality as well as the community structure of three different types of microbiomes was evaluated before and after cryopreservation with two cryoprotective agents (CPA). Microbiomes were selected based upon relevance towards applications: (1) a methanotrophic *interactome* (MOB), with potential for mitigation of greenhouse gas emissions, environmental pollutants removal and bioplastics production; (2) an oxygen limited autotrophic nitrification/denitrification (OLAND) biofilm, with enhanced economic and ecological benefits for wastewater treatment, and (3) fecal material from a human donor, with potential applications for fecal transplants and pre/probiotics research.

After three months of cryopreservation at -80°C, it was found that metabolic activity, in terms of the specific activity recovery of MOB, aerobic ammonium oxidizing bacteria (AerAOB) and anaerobic AOB (AnAOB, anammox) in the OLAND mixed culture, resumes sooner when one of the selected CPA [dimethyl sulfoxide (DMSO) and DMSO plus trehalose and tryptic soy broth (DMSO+TT)] was added. However, the activity of the fecal community was not influenced by the CPA addition, although the preservation of the community structure (as determined by 16S rRNA gene sequencing) was enhanced by addition of CPA. In summary, a cryopreservation protocol that succeeded in preserving both community structure and functionality of value-added microbiomes was established. This will allow individual laboratories and culture collections to boost the use of microbiomes in biotechnological applications.

Chapter redrafted after:

Kerckhof, F.-M.*, Courtens, E.N.P*, Geirnaert, A.*, Hoefman, S., Ho, A., Vilchez-Vargas, R., Pieper, D.H., Jauregui R., Vlaeminck, S.E., Van de Wiele, T., Vandamme, P., Heylen, K., Boon, N. Optimized cryopreservation of mixed microbial communites for conserved functionality and diversity. *PLoS ONE 2014, 9(6) e99517*

*authors contributed equally to this research

1. Introduction

In a bio-based economy, the exploitation of microbial resources represents a valuable solution for many of the current sustainability issues (Kleerebezem and van Loosdrecht 2007; Brenner, You *et al.* 2008; Possemiers, Grootaert *et al.* 2009; van der Ha, Bundervoet *et al.* 2011; Van den Abbeele, Roos *et al.* 2012; Van den Abbeele, Verstraete *et al.* 2013). Both single strains and consortia of different microorganisms with various interconnected functions (i.e. microbiomes; Read, Marzorati *et al.* (2011)) have been employed. The latter strategy has been gaining importance since the last decade (Kleerebezem and van Loosdrecht 2007; Brenner, You *et al.* 2008; Agler, Wrenn *et al.* 2011; Marshall, LaBelle *et al.* 2013; Van den Abbeele, Verstraete *et al.* 2013) and in certain applications microbiomes (*interactomes*) are known to outperform pure cultures (Dejonghe, Boon *et al.* 2001; Bell, Newman *et al.* 2005; Wittebolle, Marzorati *et al.* 2009; Hollister, Forrest *et al.* 2010; Marshall, LaBelle *et al.* 2013; Ho, de Roy *et al.* 2014). Hence, the use of mixed microbial communities is interesting both from a purely scientific point of view as well as from the viewpoint of practical applications. Nonetheless, no optimized approach to maintain a reproducible mixed community inoculum is available to date (even when correcting the inherent variability found in mixed bacterial communities (i.e. community dynamics; Read, Marzorati *et al.* (2011)).

The majority of the existing protocols for long-term and stable storage have been described for axenic cultures, which is obviously linked with the almost exclusive focus of biological resource centers (BRC) on pure culture microorganisms (Emerson and Wilson 2009; Prakash, Nimonkar *et al.* 2013). In non-BRC labs the preservation method of freezing at -80°C is preferred over freeze-drying or other drying techniques, because of the direct access to electrical freezers for most researchers and the straightforwardness of the procedure (Heylen, Hoefman *et al.* 2012). To avoid cellular damage during cryopreservation and subsequent thawing, a wide array of cryoprotective agents (CPA) has been applied. Of these, cryopreservation with dimethylsulfoxide (DMSO) is comparatively more successful than the commonly used glycerol (Hubálek 2003). Moreover, recent studies on preservation of fastidious axenic cultures have shown the effectiveness of complex media for cryopreservation of methanotrophic bacteria (MOB) (Hoefman, Van Hoorde *et al.* 2012), aerobic and anaerobic ammonia-oxidizing bacteria (AerAOB and AnAOB) (Heylen, Ettwig *et al.* 2012; Hoefman, Pommerening-Roser *et al.* 2013) and nitrite oxidizing bacteria (Vekeman, Hoefman *et al.* 2013). These complex cryopreservation media exploit the concerted protective effects of a fast penetrating CPA (DMSO) and the innate cryoprotective effects of carbon-rich

media (Trehalose and Tryptic Soy Broth, TT). Apart from the choice of CPA, which has been indicated to be one of the most determining factors for cryopreservation success (Hubálek 2003), a rigorous protocol for freezing, thawing, resuscitation and storage with as less temperature variations as possible is essential for successful cryopreservation (Heylen, Hoefman *et al.* 2012).

To date, only a few methodologies for cryopreservation of non-axenic cultures have been described (Laurin, Labbe *et al.* 2006; Vlaeminck, Geets *et al.* 2007; Hamilton, Weingarden *et al.* 2012; Heylen, Ettwig *et al.* 2012). Conversely, regardless of the increasing interest in processes driven by mixed microbial communities or microbiomes/*interactomes* (Brenner, You *et al.* 2008; Read, Marzorati *et al.* 2011), they are currently not readily available from any culture collection (Emerson and Wilson 2009). Among the described preservation methodologies, the use of DMSO and DMSO+TT as CPA has been evaluated for highly enriched anammox communities, without further evaluation of the community structure (Heylen, Ettwig *et al.* 2012). The preservation of the activity of anammox enrichments has also been evaluated at -60°C (not at -80°C) without evaluation of DMSO as a CPA but with evaluation of community changes by means of comparative FISH (Rothrock, Vanotti *et al.* 2011). Cryopreservation of gel entrapped nitrifying sludge has also been evaluated (Vogelsang, Gollembiewski *et al.* 1999) but not with DMSO as a CPA, nor with further evaluation of the community structure. The recovery of both activity and community structure (evaluated with DGGE) has been evaluated for cryopreserved denitrifying biomass (Laurin, Labbe *et al.* 2006). However, DMSO nor TT were incorporated as CPA in the study design. Finally, cryopreservation of the oxygen-limited autotrophic nitrification/denitrification (OLAND) biofilm has been evaluated previously (Vlaeminck, Geets *et al.* 2007) but cryopreservation was not evaluated at -80°C nor with the use of DMSO or DMSO+TT as a CPA. To summarize, none of the described methodologies to this day have evaluated both the documented benefits of DMSO with or without carbon rich compounds as a CPA on both community composition and functionality.

This study presents the innovative implementation of a cryopreservation protocol, designed based on previous research (Hubálek 2003; Heylen, Ettwig *et al.* 2012; Hoefman, Van Hoorde *et al.* 2012; Hoefman, Pommerening-Roser *et al.* 2013; Vekeman, Hoefman *et al.* 2013), for stable storage of bacterial mixed cultures in order to retain both community composition and an associated key functionality over time. DMSO was chosen over glycerol as the CPA. The combination of DMSO and TT was evaluated as a separate CPA. Storage was performed at -80°C. Three different bacterial mixed communities were included: (i) a

highly enriched co-culture of methane-oxidizing bacteria (MOB) and heterotrophs (van der Ha, Hoefman *et al.* 2010), (ii) a biofilm from the OLAND process (Vlaeminck, De Clippeleir *et al.* 2012) which contained both nitrifiers (aerobic ammonium-oxidizing bacteria, AerAOB and nitrite-oxidizing bacteria, NOB) and anoxic ammonium-oxidizing bacteria (or anammox bacteria: AnAOB) and (iii) a human fecal microbiome. These mixed bacterial cultures were selected based upon their relevance for science and industry. MOB mixed communities are the key drivers of a variety of biotechnological processes (Jiang, Chen *et al.* 2010): methane removal in gaseous or liquid wastestreams, production of added-value compounds from these wastestreams (Helm, Wendlandt *et al.* 2006; van der Ha, Bundervoet *et al.* 2011; Pieja, Sundstrom *et al.* 2012) or biodegradation of hazardous organic compounds (Hrsak and Begonja 2000). The OLAND mixed communities form a one-stage sustainable nitrogen removal process removing ammonia from wastewaters with a low C/N ratio and ammonia loaded gas streams through a combination of partial nitritation and anammox (Kuai and Verstraete 1998; De Clippeleir, Courtens *et al.* 2012; Vlaeminck, De Clippeleir *et al.* 2012). Finally, the fecal microbiome opens perspective for pre- and probiotics testing and fecal transplantations (Possemiers, Grootaert *et al.* 2009; Van den Abbeele, Roos *et al.* 2012; Van den Abbeele, Verstraete *et al.* 2013).

2. Materials and methods

2.1. Biomass sampling and pretreatment

Prior to cryopreservation, biomass was harvested from three different sources with their own key specific functionality.

2.1.1. MOB biomass

A methanotrophic co-culture was sub-cultivated from the original enrichment culture by Van Der Ha *et al.* (van der Ha, Hoefman *et al.* 2010) on NMS medium (with copper). Headspace air was replenished every three days, in a non-sterile fashion. Biomass was sampled from these communities growing in active methane oxidizing fed-batch reactors.

2.1.2. OLAND biomass

OLAND is a one-stage autotrophic process removing ammonia from wastewaters with a low C/N ratio and ammonia loaded gas streams through a combination of partial nitritation and anammox (Kuai and Verstraete 1998; De Clippeleir, Courtens *et al.* 2012; Vlaeminck, De Clippeleir *et al.* 2012).

The OLAND-biomass was harvested from a lab-scale rotating biological contactor (RBC) showing stable operation for several years (Pynaert, Smets *et al.* 2003). The reactor is being operated at 34±1°C and has been fed with synthetic influent at a volumetric loading rate of 600 mg N L^{-1} d^{-1} and a hydraulic residence time of 40 h. At the time of sampling, the average nitrogen removal efficiency was 77%. About 100 g of biofilm was harvested from the RBC discs by scraping. To remove all dissolved nitrogen compounds originating from the reactor liquid, the harvested biomass was washed with tap water in a sieve (pore size 250 μm).

2.1.3. Fecal biomass

Following verbal consent, a stool sample of a healthy human volunteer was collected in a sealed, plastic container with an AnaeroGen™ bag to create an anaerobic environment. The sample was preserved within 2 h after defecation.

2.2. Experimental setup

The experimental design, over a three month period of cryopreservation, is outlined in Figure 5-1. Each source of biomass at t_0 was divided in three parts: one part was subjected to cryopreservation, with or without addition of CPA, another part was subjected to the reference activity test, and a final part was sampled for DNA extraction and biomass quantification. At the end of the reference activity test, biomass was again sampled for DNA extraction and biomass quantification (t_1). After 106 days, biomass was resuscitated (t_2) and used as inoculum for the post-freezing activity test. At the end of the post-freezing activity test biomass was sampled again for quantification and DNA extraction (t_3). DNA sampling at this point allows to investigate the active community after resuscitation and a standard batch activity test.

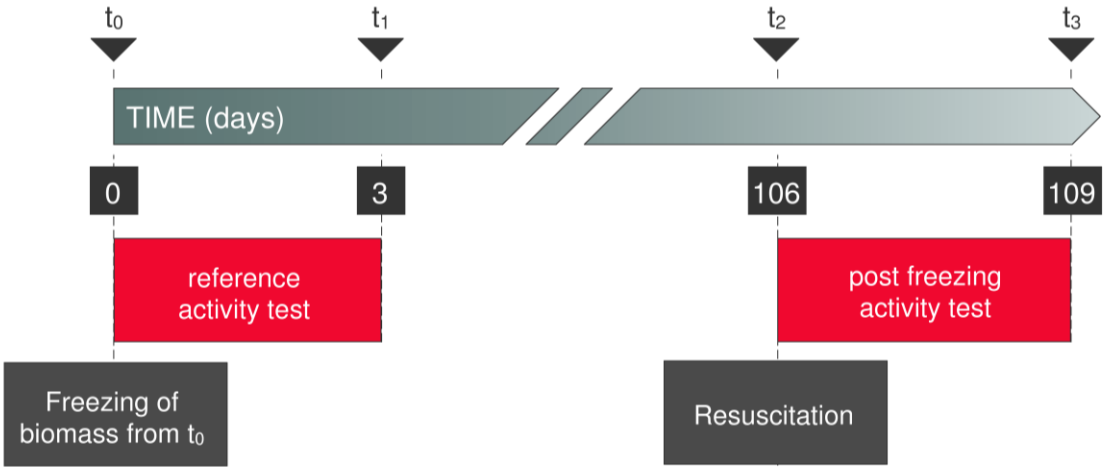


Figure 5-1. Timeline and sampling strategy of the cryopreservation setup. *The time is shown in days. In red the activity tests are shown. The freezing and thawing are shown in dark grey. DNA sampling was executed at t_0, t_1 and t_3.*

2.2.1. Storage conditions

Prior to cryopreservation and activity testing, all mixed cultures were cultivated in their appropriate growth media (described in section 2.3). Cultures were harvested from active (fed-) batch reactors or human fecal matter. Biomass was transported in under one hour to the cryopreservation location on coldpacks (approx. 4°C). Biomass was stored in quadruplicate 50 mL falcon tubes for each cryopreservation condition (see Table 5-1), to allow for an adequate amount of inoculum to be preserved to execute activity measurements and reactor startup immediately after resuscitation (Heylen, Ettwig *et al.* 2012).

In each falcon tube either 20 mL of liquid broth for the MOB biomass (previously cultivated on NMS) or 8 g of wet weight for the OLAND and fecal biomass was added. No particular precautions were made to avoid exposure of the anaerobized fecal slurry and OLAND biofilm to air in preparation of the 50 mL falcon tubes for freezing (Heylen, Ettwig *et al.* 2012). To the OLAND and fecal biomass, 20 mL of autoclaved tap water (Ghent, Belgium) or basal medium (Rechner, Smith *et al.* 2004) was added, respectively. Immediately upon arrival at the cryopreservation site, 20 mL of the selected CPA (Table 5-1), was added and gently mixed. Addition of CPA was performed at 4°C to decrease DMSO toxicity. Biomass was allowed to equilibrate with the added CPA for 30 minutes at room temperature (21°C). Immediately after, biomass was transferred to -80°C. The falcon tubes were stored in non-insulated cryopreservation boxes in an aluminum rack placed in the -80°C ULT freezer. The freezing and thawing rates in medium with 5% DMSO at -80°C were similar to values determined by the authors in a previous experiment (Hoefman, Pommerening-Roser *et al.*

2013) as an identical protocol and equipment were used. In this experiment it was shown that the rates of freezing to -80°C were much lower than in liquid nitrogen, while thawing rates were similar. A slower freezing rate is beneficial for preservation success, as a rapid cooling can increase the chance of intracellular ice formation, leading to cell death.

Table 5-1. Cryoprotective agents (CPA) used in the cryopreservation design.

Treatment designation	CPA content
No CPA	Distilled autoclaved tap water
DMSO	Distilled autoclaved tap water and DMSO to a final concentration of 5% (v/v) DMSO
DMSO+TT	TT medium (1% (w/v) D+-Trehalose, 0.3% (w/v) tryptic soy broth (TSB)) and DMSO to a final concentration of 5% (v/v) DMSO

2.2.2. Resuscitation conditions

The last step in every preservation protocol is the resuscitation of preserved biomass so that cells again become active and are able to reproduce (Heylen, Hoefman *et al.* 2012). Samples were thawed in a warm water bath at 37°C. Because of cytotoxicity of DMSO, the samples were removed from the warm water bath immediately upon thawing for centrifugation at 4°C at 7000g for 15 minutes after which the supernatant was discarded. The pellet was then resuspended in fresh medium, and 50% (v/v) TT medium was added to the corresponding vials. After resuspending, cultures were incubated for one hour at room temperature. Then the samples were centrifuged as described above following pellet resuspension in 20mL of their respective media.

2.3. Activity screening setup

2.3.1. MOB biomass

A total liquid volume of 200 mL was used to have sufficient amounts of methane and oxygen in the headspace for a 96 h incubation in 1.15 L bottles. At the start of each incubation, 20% (v/v) of methane (99.95% pure, Air Liquide, Liège, Belgium) was added to the headspace of the bottles. All cryoprotective conditions were incubated in duplicate on both NMS medium (1 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1 g L^{-1} KNO_3, 0.15 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.005 g L^{-1} FeNaEDTA, 1.43 g L^{-1} $Na_2HPO_4 \cdot 12H_2O$, 0.55 g L^{-1} KH_2PO_4 and 0.1% (v/v) of trace elements solution) with copper and dNMS medium (0.4 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.4 g L^{-1} KNO_3, 0.06 g L^{-1} $CaCl_2 \cdot 2H_2O$

and 2.87 g L^{-1} $Na_2HPO_4 \cdot 12H_2O$, 1.1 g L^{-1} KH_2PO4. Other constituents are given for NMS). The trace elements solution is described in Table 5-2.

Table 5-2. Composition of the trace element solution for NMS and dNMS with copper

Component	Concentration
$Na_2EDTA.2H_2O$	0.5 g L^{-1}
$FeSO_4.7H_2O$	0.2 g L^{-1}
H_3BO_3	0.03 g L^{-1}
$CoCl_2.6H_2O$	0.02 g L^{-1}
$ZnSO_4.7H_2O$	0.01g L^{-1}
$MnCl_2.4H_2O$	0.003 g L^{-1}
$NaMoO_4.2H_2O$	0.003 g L^{-1}
$NiCl_2.6H_2O$	0.002 g L^{-1}
$CuSO_4.5H_2O$	2.5 g L^{-1}

The bottles were placed on a shaker (120 rpm) for 48 h with daily sampling for cell dry weight (10 mL) and headspace gas (2 mL).

Specific methane oxidation rate (MOR, mmol CH_4 g^{-1} VS d^{-1}) was determined as the amount of methane consumed divided by the average volatile solids (VS) concentration over the activity test of 48h, and was the key activity for the methanotrophic *interactome*.

2.3.2. OLAND biomass

The aerobic batch experiments for AerAOB and NOB activity were performed in 250 mL Erlenmeyer flasks with 100 mL working volume, where 0.1 g L^{-1} of nitrogen added as NH_4Cl and a buffering solution (1 g L^{-1} NaHCO3, 3.4 g L^{-1} KH_2PO_4 and 4.4 g L^{-1} K_2HPO_4) were supplied to the biomass (~ 0.2g VSS per Erlenmeyer). The flasks were incubated on a shaker at 34°C while pH and dissolved oxygen concentration were monitored: samples for ammonium, nitrite and nitrate analyses were taken each 4 h. For the anoxic batch tests (AnAOB activity), 120 mL serum flasks were used, containing 80 mL of mixed liquor. Once

the biomass (~ 0.2g VSS per flask) and a buffering solution (final concentrations 1 g $NaHCO_3$ and 0.04 g L^{-1} KH_2PO_4) were added, the flasks were closed with rubber stoppers and flushed with N_2 gas (30 cycles of 800 mbar overpressure, 900 mbar underpressure). Then, flushed substrate solutions containing NH_4Cl and $NaNO_2$ were added (final concentrations 0.1 g L^{-1} NH4-N and 0.1 g L^{-1} NO_2-N). Further incubation and sampling were performed as described for the aerobic batch experiments. Due to a missing sampling point (t_1) and the pooling of the biomass, statistical analyses were not completed for the community data acquired.

2.3.3. Fecal biomass

The fresh or preserved (after removal of cryoprotectant medium) fecal sample was diluted (20%, w/v) and homogenized with sterilized phosphate buffer (0.1 M, pH 7.0), containing 1 g L^{-1} sodium thioglycolate as the reducing agent. The particulate material was removed by centrifugation (2 min, 500g) and the supernatant of this pretreatment was used as an inoculum for the batch tests.

Experiments were performed in 120 mL serum flasks flushed with N_2 (as described for the anoxic OLAND activity tests) with basal medium (Rechner, Smith *et al.* 2004). Forty mL of basal medium and 10 mL of the fecal inoculum were incubated at 37°C under continuous shaking (120 rpm) for 36 h. The activity parameter that was evaluated for the fecal community was short-chain fatty acid (SCFA) production. The total SCFA concentration was calculated as the sum of concentrations of acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, caproate and isocaproate.

2.4. Physicochemical analyses

2.4.1. Biomass quantification

In order to be able to assess specific activity, biomass concentration was assessed as cell dry weight based on a determination of total (suspended) solids (T(S)S) and volatile suspended solids (V(S)S) according to Greenberg, Eaton *et al.* (1992).

2.4.2. Headspace gas composition

At the startup and before every liquid sampling event, gas samples were taken and gas pressure was measured using a tensiometer (Infield 7 with T1Kc sensor head, UMS, München, Germany). Two mL of headspace sample was transferred to and injected on a Compact GC

(Global Analyzer Solutions, Breda, The Netherlands) equipped with one channel having a thermal conductivity detector (TCD) following a Porabond pre-column attached to a Molsieve SA column. This allowed for accurate determination of the concentration of O_2, N_2, CH_4 and CO_2 in the headspace of the cultivation reactors (van der Ha, Bundervoet *et al.* 2011).

2.4.3. Ammonium, nitrite, nitrate and SCFA analyses

Dissolved ammonium concentrations were determined by a direct colorimetrical method with the Nessler reagent at 425nm (Greenberg, Eaton *et al.* 1992). Nitrate and nitrite concentrations were analyzed using an ion chromatograph (IC 761 Compact IC, Metrohm, Herisau, Switzerland) equipped with an electrochemical conductivity detector following a Metrosep A Supp5-150 column (Metrohm, Herisau, Switzerland) and a Metrosep A Supp 4/5 guard column (Metrohm, Herisau, Switzerland). The mobile phase was 3.2 mM Na_2CO_3, 1.0 mM $NaHCO_3$ and 5 volume percent acetone at a flow rate of 0.7 mL min^{-1}. Volatile fatty acids were analyzed as described previously (De Weirdt, Possemiers *et al.* 2010): C2-C8 fatty acids (including isoforms C4-C6) were measured by gas chromatography (GC-2014, Shimadzu®, The Netherlands) with DB-FFAP 123-3232 column (30m x 0.32 mm x 0.25 µm; Agilent, Belgium) and a flame ionization detector (FID). Liquid samples were conditioned with sulfuric acid and sodium chloride and 2-methyl hexanoic acid as internal standard for quantification of further extraction with diethyl ether. Prepared sample (1 µL) was injected at 200ºC with a split ratio of 60 and a purge flow of 3 mL min^{-1}. The oven temperature increased by 6ºC min^{-1} from 110ºC to 165ºC where it was kept for 2 min. FID had a temperature of 220ºC. The carrier gas was nitrogen at a flow rate of 2.49 mL min^{-1}.

2.5. Microbial identification and quantification

To identify the mixed bacterial community constituents, Illumina 16S rRNA gene sequencing was performed. DNA was extracted using the FastDNA™ SPIN kit for soil (MP Biomedicals, Brussels, Belgium) according to the instructions of the manufacturer for the MOB and OLAND samples and by using the CTAB method (Griffiths, Whiteley *et al.* 2000) for the human fecal material samples.

The preparative amplification, gel purification and equimolar pooling for Illumina amplicon sequencing was performed as described before (Camarinha-Silva, Jáuregui *et al.* 2014). Unidirectional Illumina amplicon sequencing was executed with 16S rRNA gene primers for

the V5-V6 hypervariable regions as described before (Bohorquez, Delgado-Serrano *et al.* 2012).

A total of 544568, 83754 and 170617 sequence reads were obtained for the 20 MOB samples, 4 OLAND samples and 9 fecal microbiome samples, respectively. A quality filter program that runs a sliding window of 10% of the read length over the read and calculates the local average score based on the Phred quality scores of the fastq file, trimmed 3'-ends of the reads that fall below a quality score of 15 (http://bioinformatics.ucdavis.edu/index.php/Trim.pl). Only reads of a minimum of 149 nt in length (29 nt of primer and barcode sequence and 120 nt of 16S rRNA gene sequence) were further analyzed. All truncated reads that had an N character (ambiguous base call) in their sequence, any mismatches within primers and barcodes or more than 10 homopolymer stretches were discarded. All sequences from each sample present in the different libraries were split into different files according to their unique barcode.

A representative read was further considered if a) it was present in at least one sample in a relative abundance >1% of the total sequences of that sample or b) was present in at least 2% of samples at a relative abundance >0.1% or c) present in at least 5% of samples. Phylotype representatives were then generated by clustering at 98% similarity (1 mismatch) using the mothur pre.cluster program (Schloss, Westcott *et al.* 2009). This reduced the number of representative reads to a computational manageable level without curtailing the fine scale community composition (Camarinha-Silva, Jáuregui *et al.* 2014). The final sequences used for this study are available as supplemental material (http://journals.plos.org/plosone/article?id=10.1371%2Fjournal.pone.0099517#s6).

2.6. Data processing and visualization

All statistical data analyses and graphing of community structure were performed with the statistical software R, version 3.0.2. for Windows (http://www.r-project.org) (R Development Core Team 2015). Multiple comparisons were executed using the Kruskal-Wallis rank sum test from the R base package stats. If the null hypothesis of equality of location parameters of each group distribution was rejected, nonparametric relative contrast effects (Konietschke 2009; Konietschke, Placzek *et al.* 2015) were estimated to assess significant differences between groups with Tukey contrasts, unless stated otherwise. Graphing of the functionality data was performed using SigmaPlot for Windows version 12.0 (Systat software, Inc.).

Phylogenetic trees were constructed after sequence alignment using mothur, version 1.31.2 (Schloss, Westcott *et al.* 2009). Alignments were made using the align.seqs command with the reference Silva alignment provided on the mothur website (Release 102, http://www.mothur.org/wiki/Silva_reference_files). RAxML (Stamatakis 2006; Stamatakis 2014) was used to construct a majority rule bootstrap consensus tree with the GTR+GAMMA substitution model. One thousand bootstrap iterations were executed using the parallelization offered in the Pthreads-based version of RAxML (Ott, Zola *et al.* 2010). The Newick-formatted output tree was subsequently loaded into iTol (http://itol.embl.de) for data visualization (Letunic and Bork 2011). Classification of sequences was executed with the mothur implementation of the naïve Bayesian classifier (Wang, Garrity *et al.* 2007) with a threshold of 0.65 with either RDP release 9 (Cole, Wang *et al.* 2009) reference taxonomy or the Greengenes reference taxonomy suggested by Werner and colleagues (Werner, Koren *et al.* 2012), without trimming to the sequencing region. Unless stated otherwise, the sequence count data were randomly subsampled to the sequence count of the sample with the lowest sequence count for each of the mixed communities separately. Rarefaction curves showed that at this cutoff all samples were sequenced deep enough to cover biodiversity (Figure 5-2).

When means are reported, they are always reported as mean ± standard deviation ($\hat{\mu} \pm \hat{\sigma}$) and the number of replicates (*n*) is given between brackets. Multiple comparisons were made using nonparametric relative contrast effects (Konietschke and Hothorn 2012) with Tukey contrasts and a logit asymptotic approximation as implemented in the R package nparcomp (Konietschke, Placzek *et al.* 2015) unless otherwise stated.

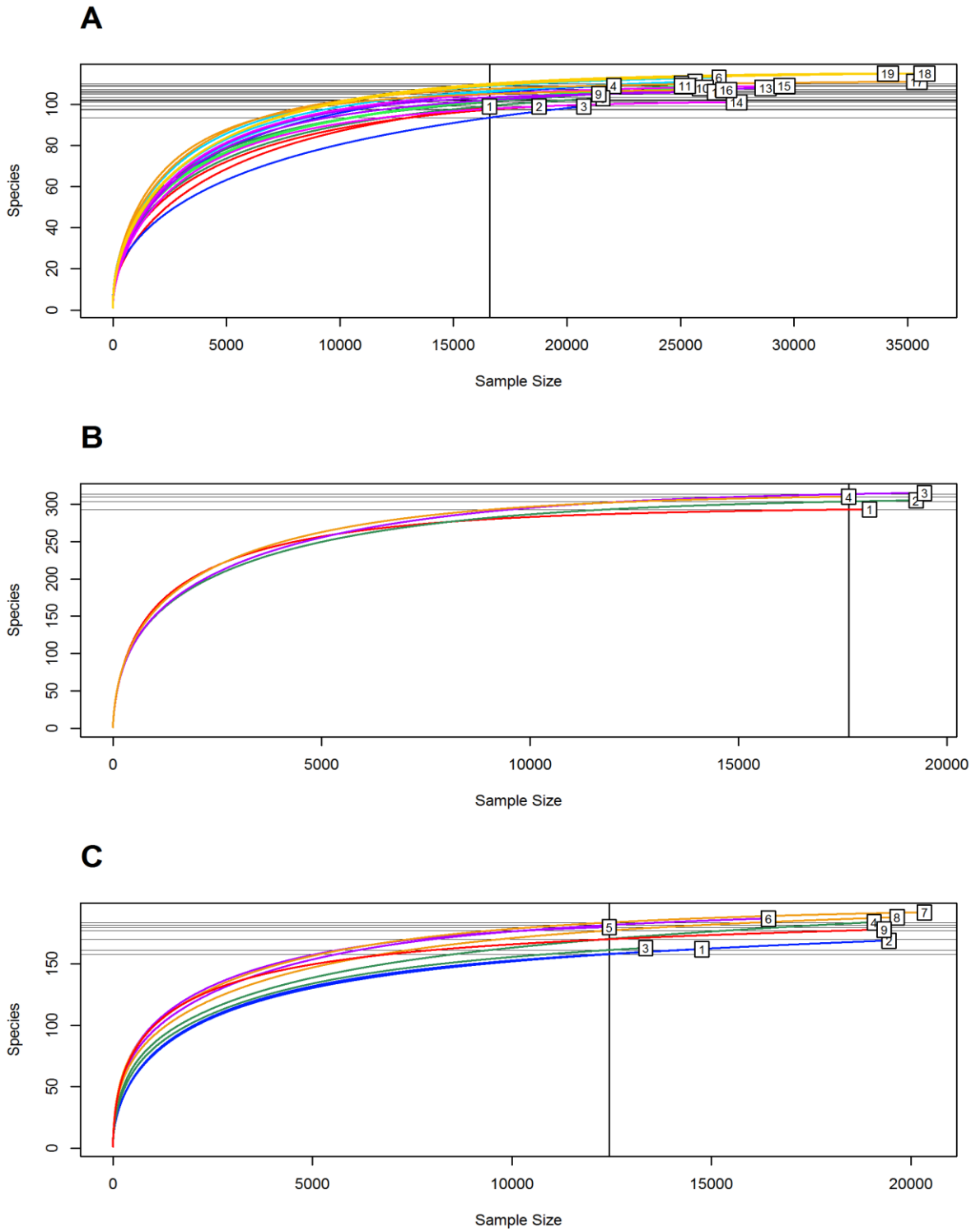


Figure 5-2. Rarefaction curves. *Colors represent the sample types as indicated in the phylogenetic trees in* ***Figure 5-4***, ***Figure 5-8*** *and* ***Figure 5-10****. (A) MOB community rarefaction. Samples 1 & 2: inoculum (t_0), 3&4 : NMS t_1, 5-7 : dNMS t_1, 8&9 : No CPA NMS, 10&11 : No CPA dNMS, 12 & 13 : DMSO NMS, 14& 15 : DMSO dNMS, 16 & 17 : DMSO+TT NMS, 18&19 : DMSO+ TT dNMS. The dataset was subsampled to 16591 reads per sample. (B) OLAND community rarefaction. Pooled samples 1: t_0, 2: No CPA, 3: DMSO, 4: DMSO+TT. The dataset was subsampled to 17647 reads per sample. (C) Fecal microbiome rarefaction. 1&2: t_1, 3&4: No CPA, 5&6 DMSO, 7&8 DMSO+TT, 9: fecal inoculum (t_0). The dataset was subsampled to 12440 reads per sample.*

3. Results

For each of the three mixed communities the impact of the addition of a CPA during cryopreservation is outlined below. First specific activity recovery of a key activity of the mixed culture is given, followed by the results of the recovery of OTUs directly contributing to the chosen key activity (functional community members) after which the recovery of every single OTU, regardless of classification as a functional community member, is evaluated for their presence or absence at each time point and condition of the experimental design (Figure 5-1). Finally the changes in community structure are evaluated using the abundance-based Jaccard index.

3.1. Methanotrophic community (MOB)

Two cultivation media (NMS and dNMS) were evaluated with the MOB mixed culture. The key specific activity (MOR) was comparable to the original activity, when a CPA was added prior to cryopreservation (Figure 5-3A), on both media. When no CPA was added, the average specific activity over 48h was significantly lower than the original activity on both media ($p<0.0001$). The largest activity recovery was obtained when only DMSO was added as a CPA both with NMS (147.2±2.6%) and dNMS (156.1±10.1%), which exceeded the original activity. When DMSO+TT was used as a CPA, the original specific activity was obtained on NMS (96.4±10.7%) and dNMS (116.5±20%). Both on NMS and dNMS with DMSO+TT as a CPA the specific activity was not significantly different from the initial activity ($p=1$).

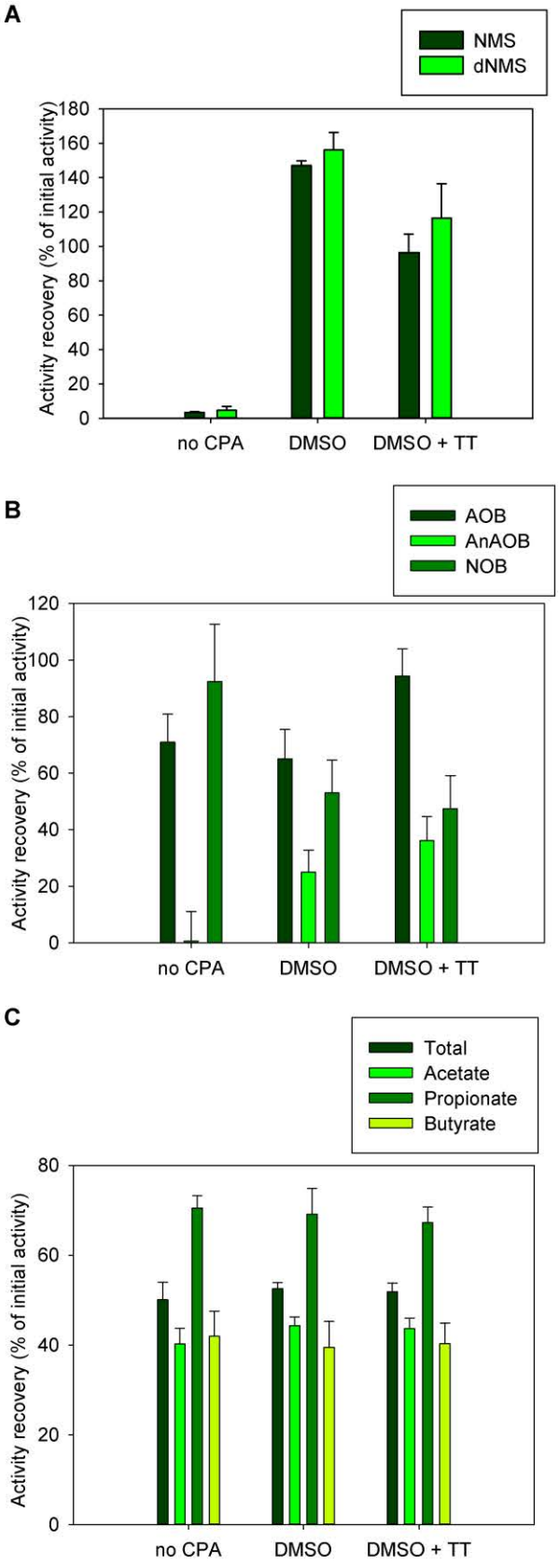
Among the OTUs classified as methylotrophic *Proteobacteria* (57 out of 117 OTUs) were classified into two MOB families (7 out of 117 OTUs): either *Methylococcaceae* (Gammaproteobacteria) or *Methylocystaceae* (Alphaproteobacteria). Also one OTU was classified as a non-proteobacterial MOB, part of the *Verrucomicrobia* phylum, namely *Candidatus Methylacidiphilum*. While the *Methylococcaceae* family was an abundant community constituent (up to 46% of all sequences of the inoculum), the *Methylocystaceae* and *Candidatus Methylacidiphilum* were less abundant (Figure 5-5, Figure 5-6). All of these methanotrophic taxa were detected in the mixed culture before and after cryopreservation, irrespective of the added CPA (Figure 5-5). However, the relative abundances of the MOB OTUs among all experimental conditions simultaneously (t_0,t_1 and t_3) were significantly different for each MOB family ($p<0.0001$). More specifically, the relative abundance of *Methylococcaceae* within dNMS pre (t_1) and dNMS DMSO (t_3) did not significantly differ from the inoculum (t_0, $p=1$). There was also no significant difference in *Methylococcaceae*

relative abundance between the dNMS pre (t_1) and dNMS samples at t_3 with DMSO and DMSO+TT (p=1). However, on NMS, all conditions at t_3 differed significantly from t_0 and t_1, regardless of CPA addition (p<0.0001). There were no significant differences in relative abundance of *Methylocystaceae* between t_3 and the initial inoculum (t_0, p=1), although the NMS samples showed a significant difference between t_3 and t_1 in samples with CPA addition (p<0.0001). No significant differences (p=1) were found in the relative abundance of *Candidatus Methylacidiphilum* between t_0 on t_3 only when DMSO was used as a CPA and incubation was performed on NMS. Between t_1 and t_3 on NMS no significant differences were found when DMSO+TT or no CPA was used (p=1). On dNMS, DMSO+TT maintains the relative abundance between t_1 and t_3 (p=1).

The main constituents of the mixed MOB culture were from the *Methylophilaceae*, *Flavobacteriaceae*, *Methylococcaceae*, *Comamonadaceae*, *Verrucomicrobiaceae*, *Chitinophagaceae* and *Enterobacteriaceae* families (Figure 5-4). Differences in relative abundance of individual community members were apparent however; only 21% of OTUs (representing only 2.5% of total sequence counts) were not detected in at least one of the experimental conditions (Figure 5-4).

Cryopreservation was associated with greater community dissimilarities on NMS, regardless of the addition of a CPA (Figure 5-13A). Nonetheless, these dissimilarities (t_0-t_3) were not significantly different from the community dissimilarity of the first activity test (t_0-t_1, p=0.12). With dNMS, the differences were significant (p=0.01). More specifically, the dissimilarity with the inoculum was lower when a CPA was added. This dissimilarity was within the range of the dissimilarity of the pre-freezing activity activity test (t_0–t_1) when DMSO was used as a CPA (Figure 5-13). The community dissimilarities in the reference (t_0–t_1), DMSO (t_1–t_3) and DMSO+TT (t_1–t_3) samples were not significantly different ($p_{ref\text{-}DMSO}=0.99$, $p_{ref\text{-}DMSO+TT}=0.50$, $p_{DMSO\text{-}DMSO+TT}=0.57$) while the dissimilarities between the reference and the samples with no CPA did significantly differ (p<0.0001).

Figure 5-3. Functionality recovery after cryoporeservation. *Error bars represent standard errors. A) the MOB community on NMS and dNMS cultivation medium. The activity recovery was the percentage of specific MOR (mmol CH_4 g^{-1} VS d^{-1}) from the pre-freezing activity test (t_0 to t_1) that was obtained in each experimental condition in the post-freezing activity test (t_2 to t_3). B) The OLAND community. The activity recovery was the percentage of specific activity (mg N g^{-1} VSS d^{-1}) for either aerobic or anaerobic ammonium oxidation (AOB and AnAOB) or nitrite oxidation (NOB), from the pre-freezing activity test (t_0 to t_1) that was obtained in each experimental condition in the post-freezing activity test (t_2 to t_3). C) short chain fatty acid production by the fecal microbiome. The activity recovery was the percentage of SCFA produced in the pre-freezing activity test (t_0 to t_1) that was obtained in each experimental condition in the post-freezing activity test (t_2 to t_3). Total SCFA, acetate, propionate and butyrate were measured.* ***Figure on next page.***


A
NMS
dNMS
Activity recovery (% of initial activity)
0
20
40
60
80
100
120
140
160
180
no CPA
DMSO
DMSO + TT
B
AOB
AnAOB
NOB
Activity recovery (% of initial activity)
0
20
40
60
80
100
120
no CPA
DMSO
DMSO + TT
C
Total
Acetate
Propionate
Butyrate
Activity recovery (% of initial activity)
0
20
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80
no CPA
DMSO
DMSO + TT

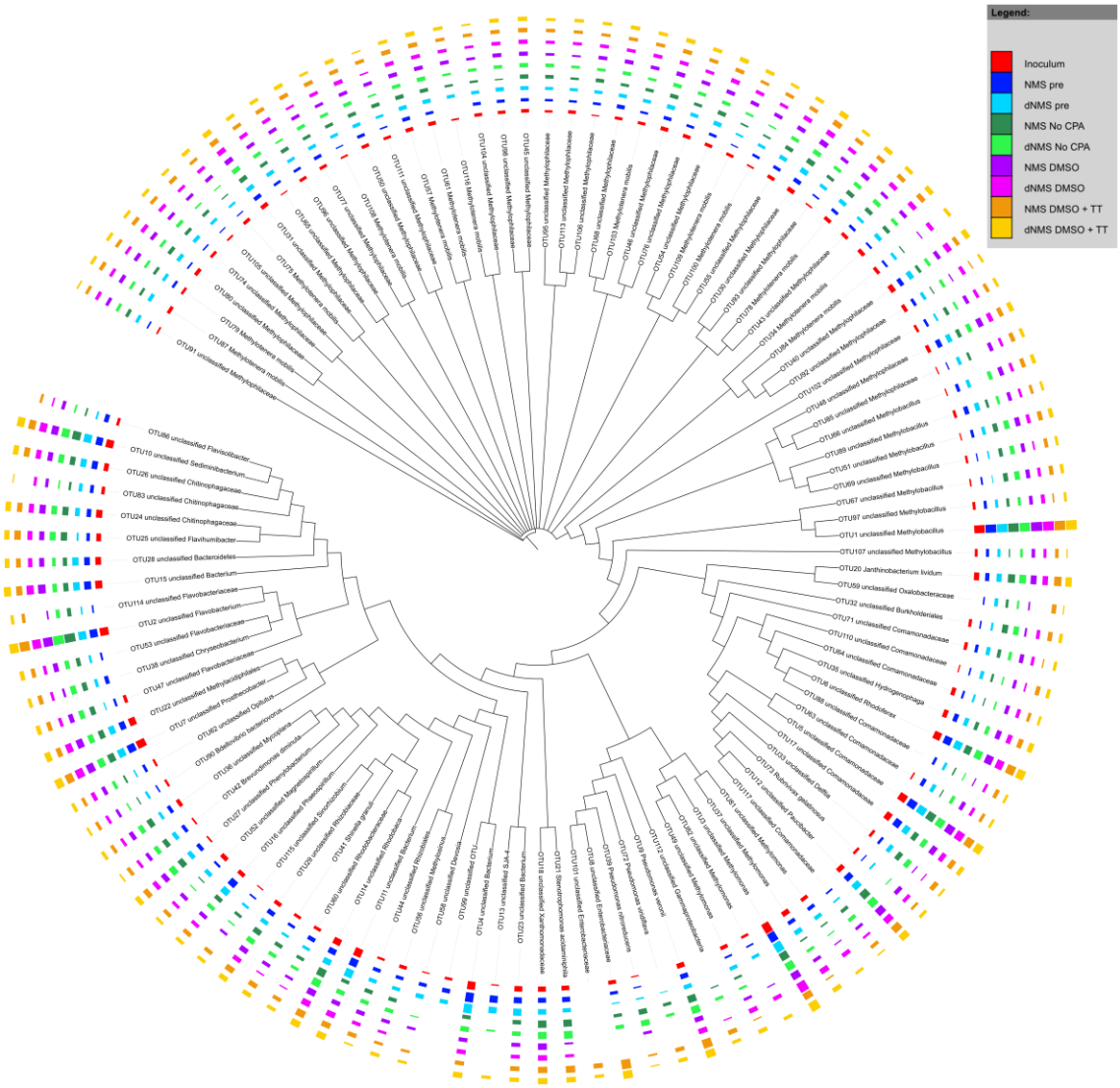

Figure 5-4. Phylogenetic tree of OTU consensus sequences in the MOB samples. *Sequences were aligned using the mothur implementation of the NAST algorithm with the Silva v102 reference alignment. RAxML was used to construct an extended majority rule bootstrap consensus tree with the GTR + GAMMA substitution model and 1000 bootstrap iterations. This bootstrap consensus tree was visualized using iTol. The colored bars represent treatment-wise means (n=2 except for inoculum n=1 and NMS pre n=3) of the log transformed absolute abundances with the log transformation as suggested by Anderson and colleagues (Anderson, Ellingsen et al. 2006) with base 10. Before transformation the samples were rarefied to the lowest sequence count after removal of anomalous sample NMS1. Classification was done based upon the Greengenes taxonomy (adapted to mothur from (Werner, Koren et al. 2012)) with the naïve Bayesian classifier implemented in mothur (Wang algorithm).*

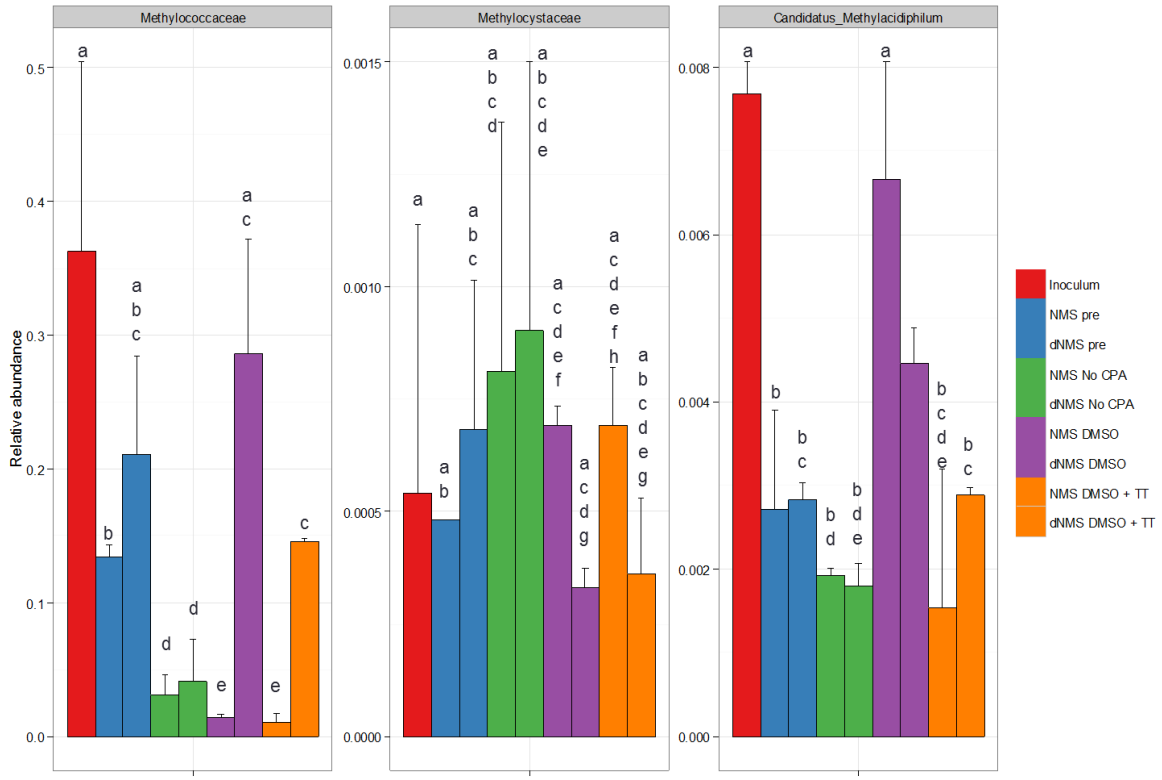

Figure 5-5. Relative abundance of methanotrophic families within the rarefied dataset. *Bar heights represent means of duplicate (Inoculum and all post samples) or triplicate (pre samples) reactors. Error bars represent the respective standard deviations. Bars with equal letters are not significantly different at the 95% significance level.*

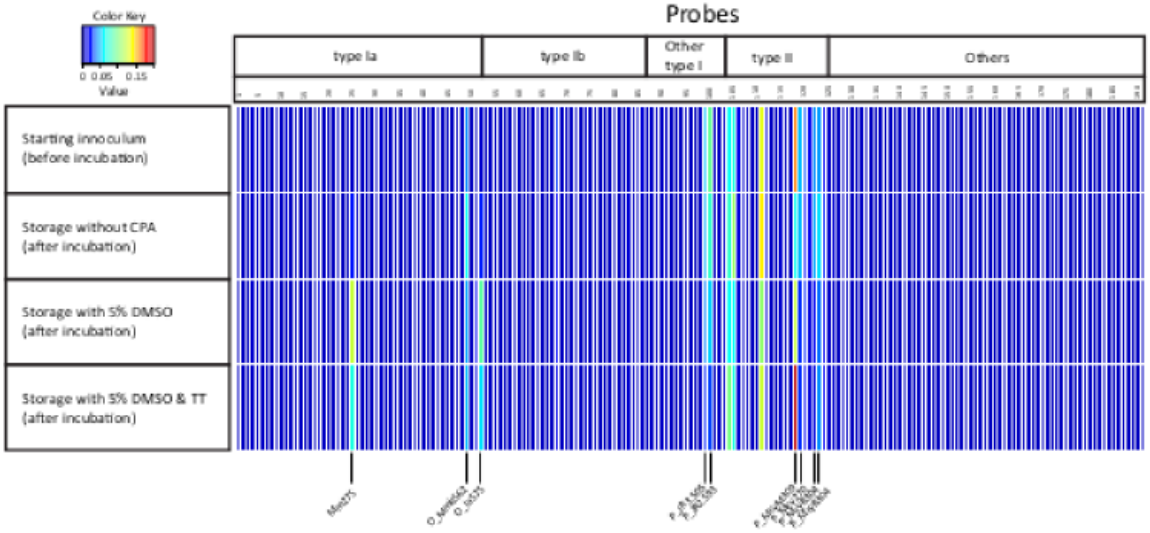

Figure 5-6. MOB diversity microarray results.

Overall, partial constrained correspondence analysis (pCCA) showed that the global community structure of samples at t_3 with an added CPA is closer to the inoculum (t_0) and results after the first incubation (t_1) and significantly ($p=0.01$) correlates with an increased recovery of MOR (Figure 5-7). This analysis 'partials' out the effect of the medium and allows to observe only the effect of CPA addition.

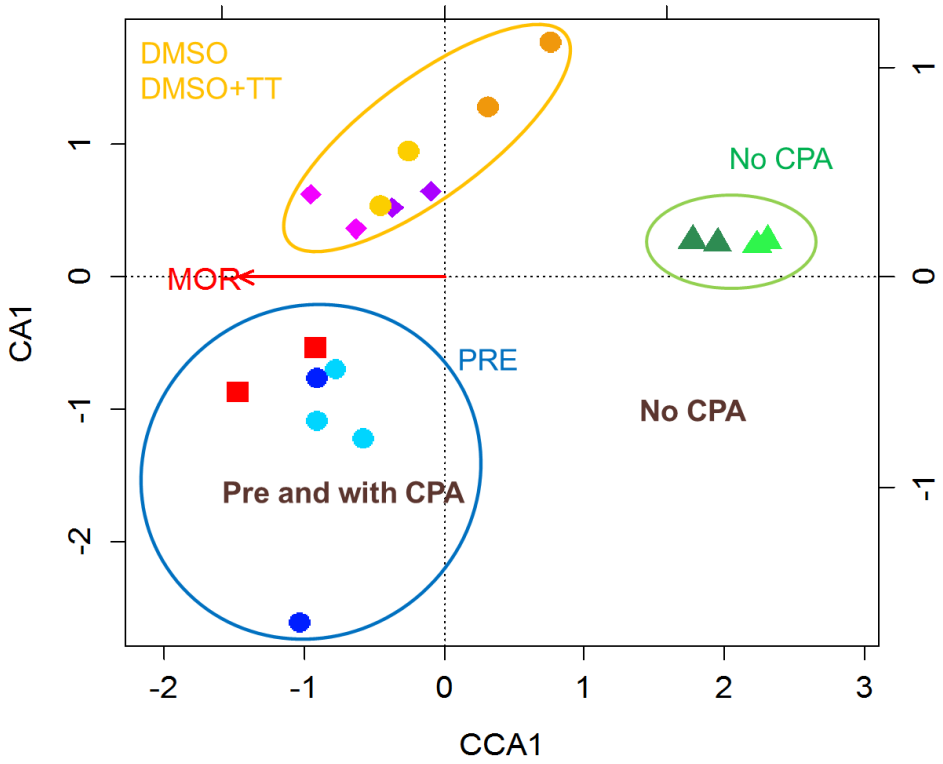

Figure 5-7. Partial Constrained Correspondence Analysis ((p)CCA) ordination graph for the MOB community. *The analysis was constrained (26% of total inertia) on the MOR ($p = 0.01$) and conditioned (2% of total inertia) on media (NMS/dNMS). The red arrow represents increasing MOR. Shapes with a dark color represent samples incubated with NMS whilst shapes with a light color represent samples incubated on dNMS. The green triangles correspond to the samples cryopreserved without CPA (t_3). Orange/gold circles represent samples cryopreserved with DMSO+TT (t_3). Purple diamonds represent samples cryopreserved with only DMSO as a CPA (t_3). Blue circles represent samples after the reference activity test (t_1) and red squares represent the original inoculum (t_0). Clusters of samples are highlighted. The distance between individual samples was calculated based upon the abundance-based Jaccard index*

3.2. OLAND mixed community

The functional autotrophic microorganisms in OLAND include AerAOB and AnAOB (Figure 5-3B). AerAOB had a complete specific activity recovery when DMSO+TT was used as a CPA (94.4±9.6%, p=1) whilst only 71.0±9.9% was obtained without addition of CPA and only 65.1±10.4% was obtained when DMSO without TT was used as a CPA. AnAOB activity

was recovered up to 36.1±8.6% with DMSO+TT as a CPA and up to 25.0±7.7% with only DMSO as a CPA. The initial specific activity was not recovered without CPA addition (0.7±10.4%). Finally NOB activity was best retained when no CPA was added (92.4±20.3%, p=0.95) whilst only 53.1±11.6% or 47.4±11.7% was recovered with DMSO and DMSO+TT, respectively.

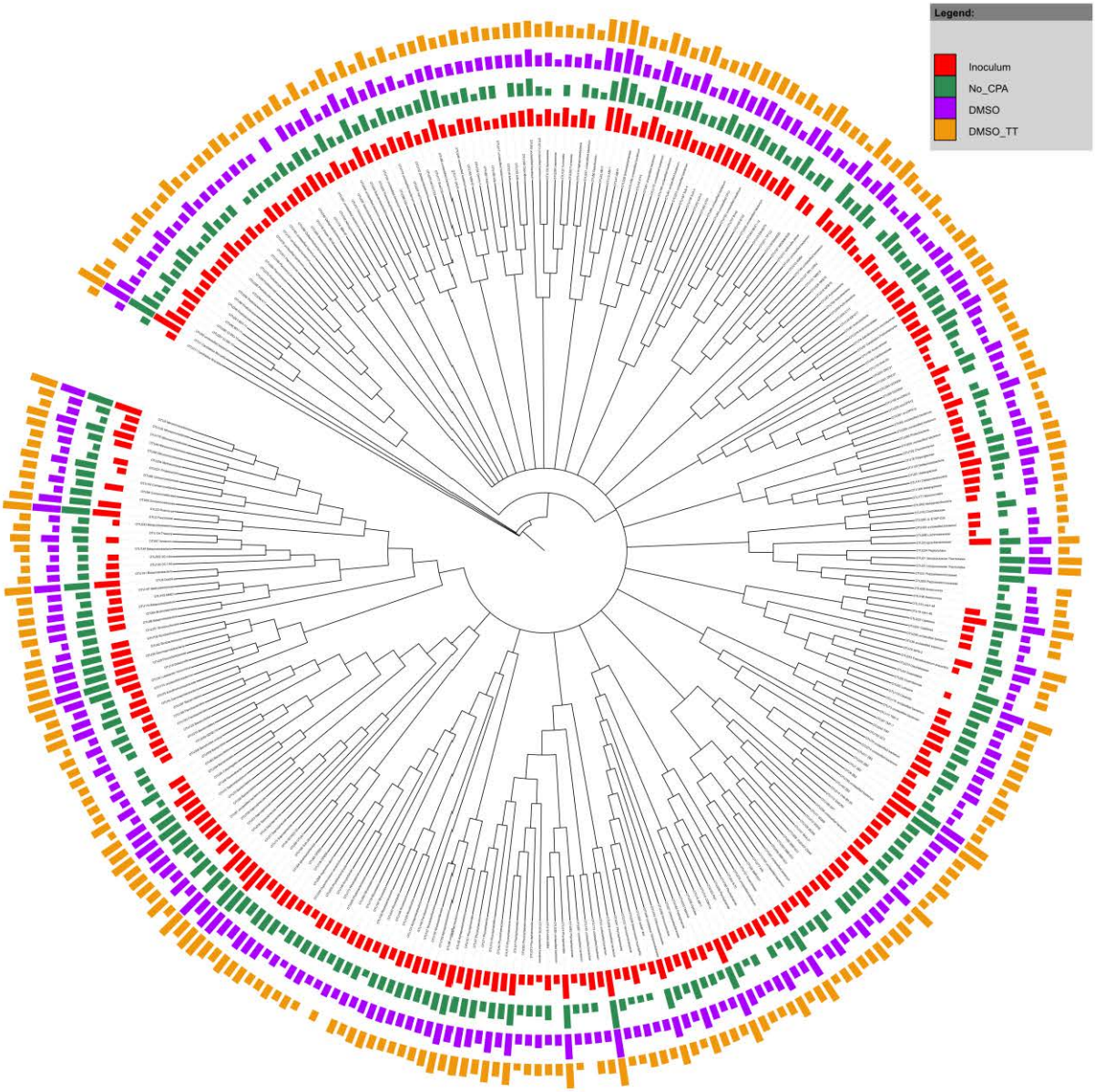

Figure 5-8. Phylogenetic tree of OTU consensus sequences in the OLAND samples. *Sequences were aligned using the mothur implementation of the NAST algorithm with the Silva v102 reference alignment. RAxML was used to construct an extended majority rule bootstrap consensus tree with the GTR + GAMMA substitution model and 1000 bootstrap iterations. This bootstrap consensus tree was visualized using iTol. The colored bars represent log transformed absolute abundances with the log transformation as suggested by Anderson and colleagues (Anderson, Ellingsen et al. 2006) with base 10. Before transformation the samples were rarefied to the lowest sequence count.. Classification was done based upon the Greengenes taxonomy (adapted to mothur from Werner, Koren et al. (2012)) with the naïve Bayesian classifier implemented in mothur (Wang algorithm).* ***Figure on previous page.***

All OTUs that could be classified as AerAOB were representatives of the *Nitrosomonadaceae* family, more specifically *Nitrosomonas* sp.. The AnAOB-OTUs were represented by the *Brocadiaceae* family, and more specifically by *Candidatus* Brocadia sp.. NOB were representatives of *Nitrospiraceae* family, more specifically *Nitrospira* sp.. Overall, the conditions where DMSO+TT was added as a CPA allowed for the best recovery after cryopreservation of all of the OLAND "functional" partners. The differences in relative abundance between no CPA and DMSO were rather minute (Figure 5-9).

The main constituents of the OLAND mixed community were representatives of the *Comamonadaceae*, *Flavobacteriaceae*, *Nitrosomonadaceae*, *Rhodocylaceae* and *OD1 incertae sedis* families as well as the *Bacteroidetes* order (Figure 5-8). Overall, most OTUs occurred in each condition irrespective of CPA addition, and only 15% of OTUs (representing 5.3% of total sequences) were not detected in at least one of the experimental conditions (Figure 5-8). Most of the OTUs that did not occur in every condition required the addition of a CPA to persist after cryopreservation. In the samples where a CPA was added, global community dissimilarity to the inoculum was lower, regardless of the type of CPA used (Figure 5-13B).

3.3. Fecal microbiome

The overall SCFA concentration profile remained nearly identical (Permutation Hotelling T^2 p-value: 0.69) between t_1 and t_3 (Figure 5-3C), although the total concentration was significantly lower ($p<0.0001$). Initial metabolic activity was reduced to 50.1±3.9% if no CPA was employed, and to 52.5±1.4% with DMSO or to at 51.9±1.9% with DMSO+TT ($p=0.58$). This decrease in total SCFA levels primarily originated from a decrease in acetate from 25.4±0.7mM (n=2) to 10.2±0.7mM (n=4) without CPA ($p<0.0001$), 11.2±0.6mM (n=3) on DMSO ($p<0.0001$) and 11.1±0.8mM (n=4) on DMSO+TT ($p<0.0001$). Compared to the

initial levels (4.98±0.01mM (n=2)) propionate levels were highest without CPA addition (70.5±2.8%, 3.5±0.1mM (n=4)) and only slightly lower with DMSO (69.1±5.7%, 3.4±0.3mM (n=3), p=0.51) or DMSO+TT (67.3±3.5%, 3.4±0.2mM (n=4), p=0.87). Finally, the concentration of butyrate was highest with DMSO+TT (1.8±0.2mM (n=4)), which was 42.0±5.5% of the initial 4.2±0.07mM (n=2) while DMSO and no CPA resulted in 1.7±0.2mM (n=3) and 1.7±0.2mM (n=4), respectively.

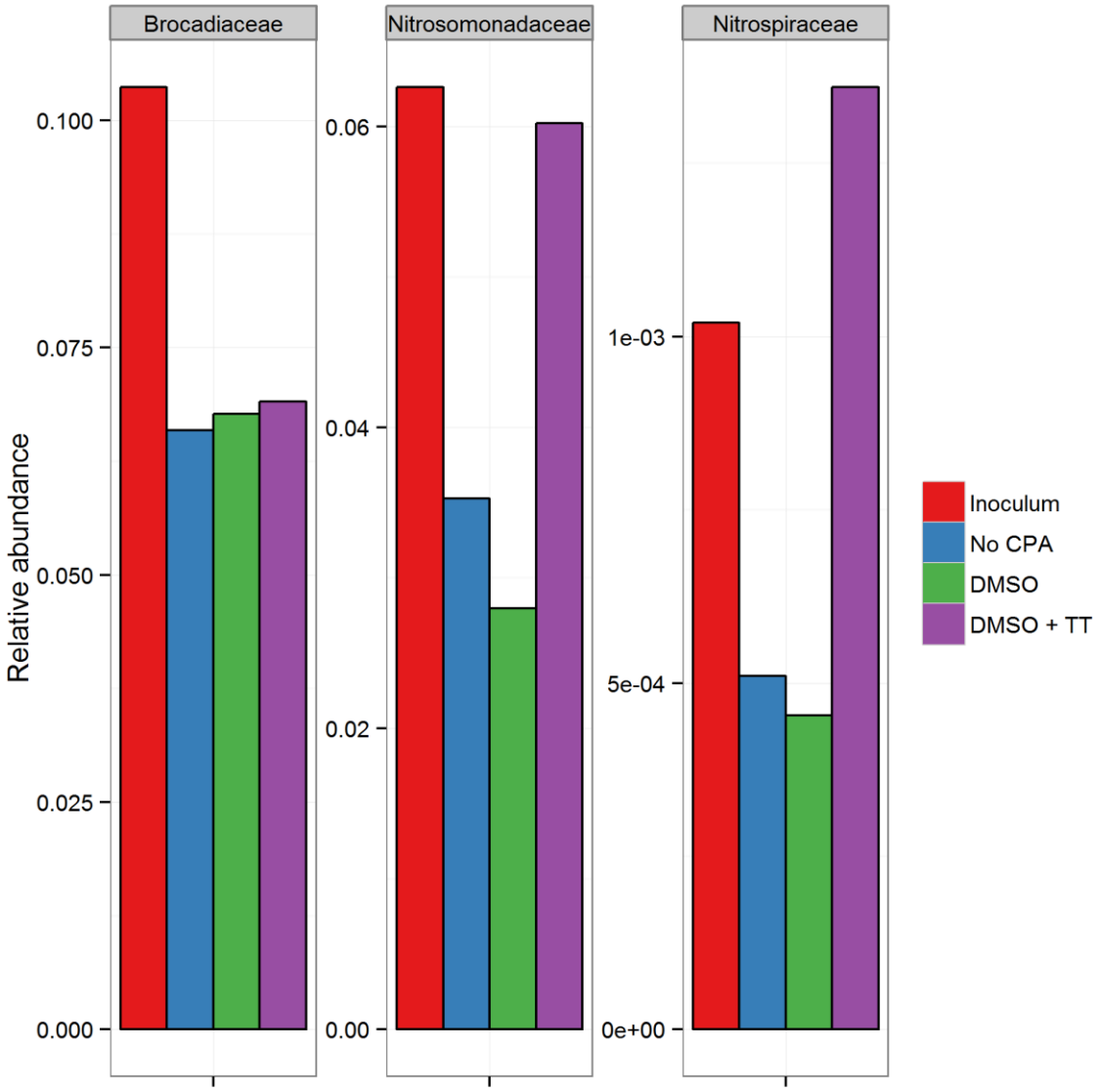

Figure 5-9. Relative abundance of relevant families for the OLAND process within the rarefied dataset.

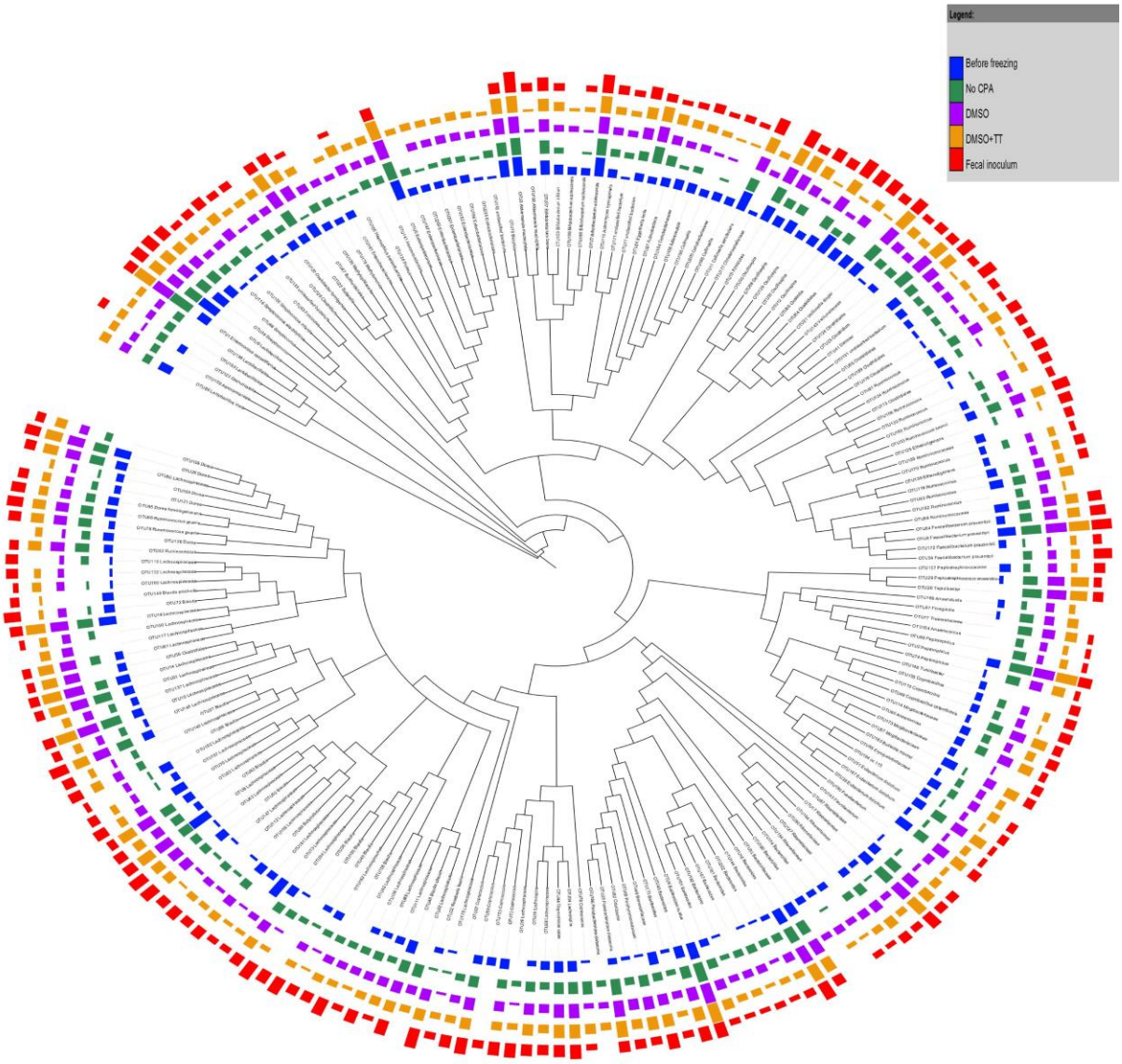

Figure 5-10. Phylogenetic tree of OTU consensus sequences in the fecal material samples. *Sequences were aligned using the mothur implementation of the NAST algorithm with the Silva v102 reference alignment. RAxML was used to construct an extended majority rule bootstrap consensus tree with the GTR + GAMMA substitution model and 1000 bootstrap iterations. This bootstrap consensus tree was visualized using iTol. The colored bars represent treatment-wise means (n=2 except for fecal inoculum n=1) of the log transformed absolute abundances with the log transformation as suggested by Anderson and colleagues (Anderson, Ellingsen et al. 2006) with base 10. Before transformation the samples were rarefied to the lowest sequence count. Classification was done based upond the Greengenes taxonomy (adapted to mothur from Werner, Koren et al. (2012)) with the naïve Bayesian classifier implemented in mothur (Wang algorithm).*

The most abundant microorganisms in the fecal microbiome were representatives of the *Lachnospiraceae*, *Bacteroidaceae*, *Ruminococcacae*, *Enterococcaceae*, *Enterobacteriaceae*, *Verrucomicrobiaceae*, *Bifidobacteriaceae* and *Clostridiales Family XI. Incertae sedis* families (Figure 5-10). A total of 18 different families, with documented associations with the fermentative metabolism in the gut, were investigated for their relative abundance before and after cryopreservation (Figure 5-11). Variable results were obtained in the different taxonomic groups. Similar to the MOB and OLAND mixed community, most OTUs from the fecal microbiome remained present in all experimental stages and conditions, irrespective of CPA addition (Figure 5-10). Of all observed OTUs, 29% (representing 8.5% of total sequences) were not detected in at least one of the experimental conditions (pre-freezing, post-freezing with or without CPA). This percentage excludes the OTU presence in the fecal inoculum due to the drastic change of the community upon first cultivation. Addition of CPA during cryopreservation of the fecal biomass was necessary to maintain comparable community dissimilarities to the reference activity test (Figure 5-13D), but differences in community dissimilarity between the conditions with and without CPA were not significant ($p=0.24$).

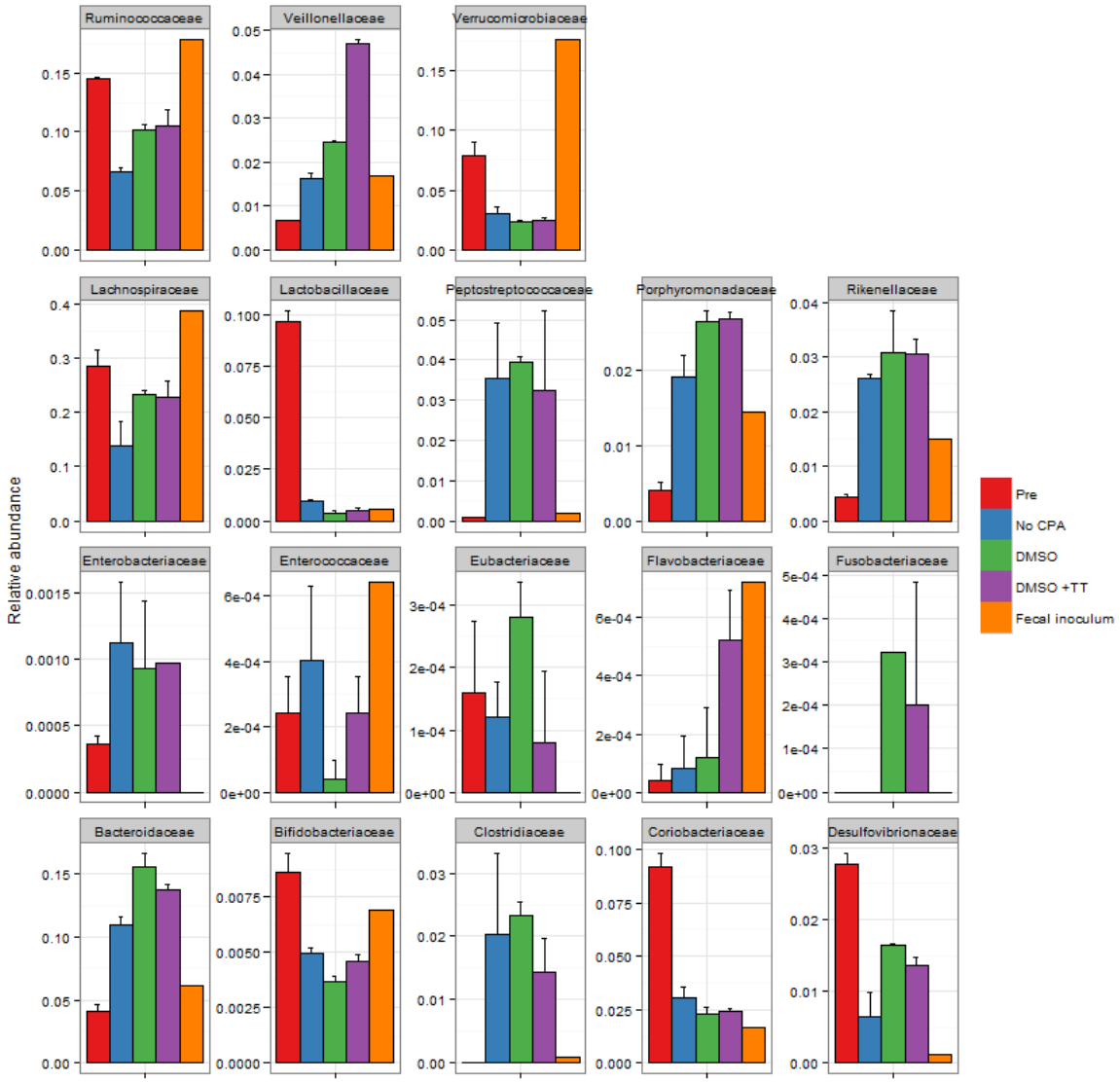

Figure 5-11. Relative abundance of relevant families for the SCFA production within the fecal community within the rarefied dataset. *Classification was done with the SILVA v111 database and the SINA aligner. Axes are not constant. The error bars represent the standard deviation of biological duplicate incubations. The fecal inoculum is displayed, as a reference (n = 1).*

Overall, constrained correspondence analysis (CCA) showed that the global community structure of samples with an added CPA at t_3 is moderately closer to the samples after the first incubation (t_1). No significant correlations with VFA production were found with marginal effects permutation tests however with sequential effects a significant correlation was found with an increased recovery of acetic acid production (Figure 5-12).

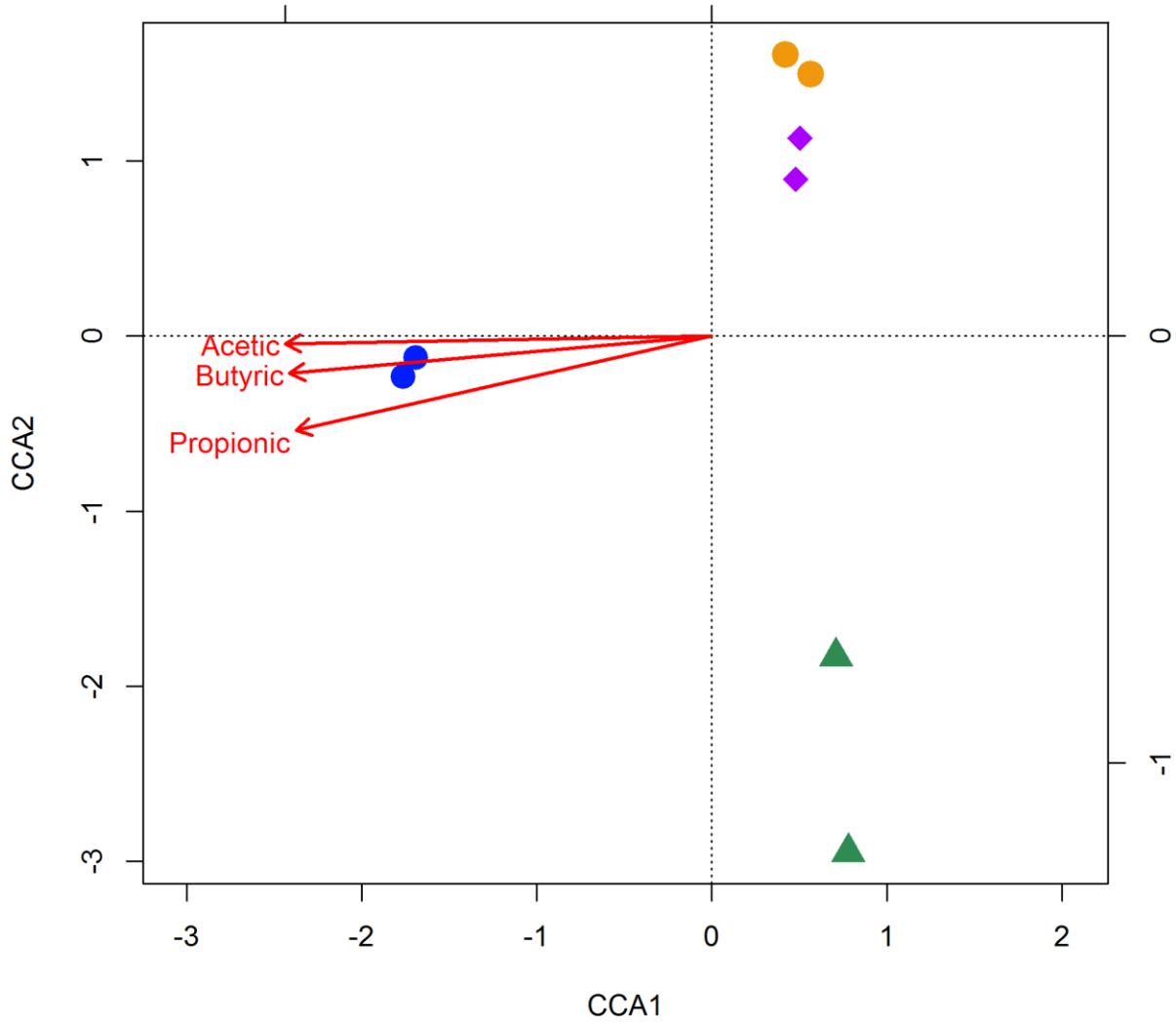

Figure 5-12. Constrained Correspondence Analysis (CCA) ordination graph for the fecal community. *The fecal inoculum was removed from the analysis. The analysis was constrained (81% of total inertia) on the concentrations of acetic acid (p = 0.02), propionic acid (p = 0.37) and butyric acid (p = 0.76). The red arrows represent increasing SCFA concentrations. The green triangles correspond to the samples cryopreserved without CPA (t_3). Orange/gold circles represent samples cryopreserved with DMSO+TT (t_3). Purple diamonds represent samples cryopreserved with only DMSO as a CPA (t_3). Blue circles represent samples after the reference activity test (t_1) and The distance between individual samples was calculated based upon the abundance-based Jaccard index.*

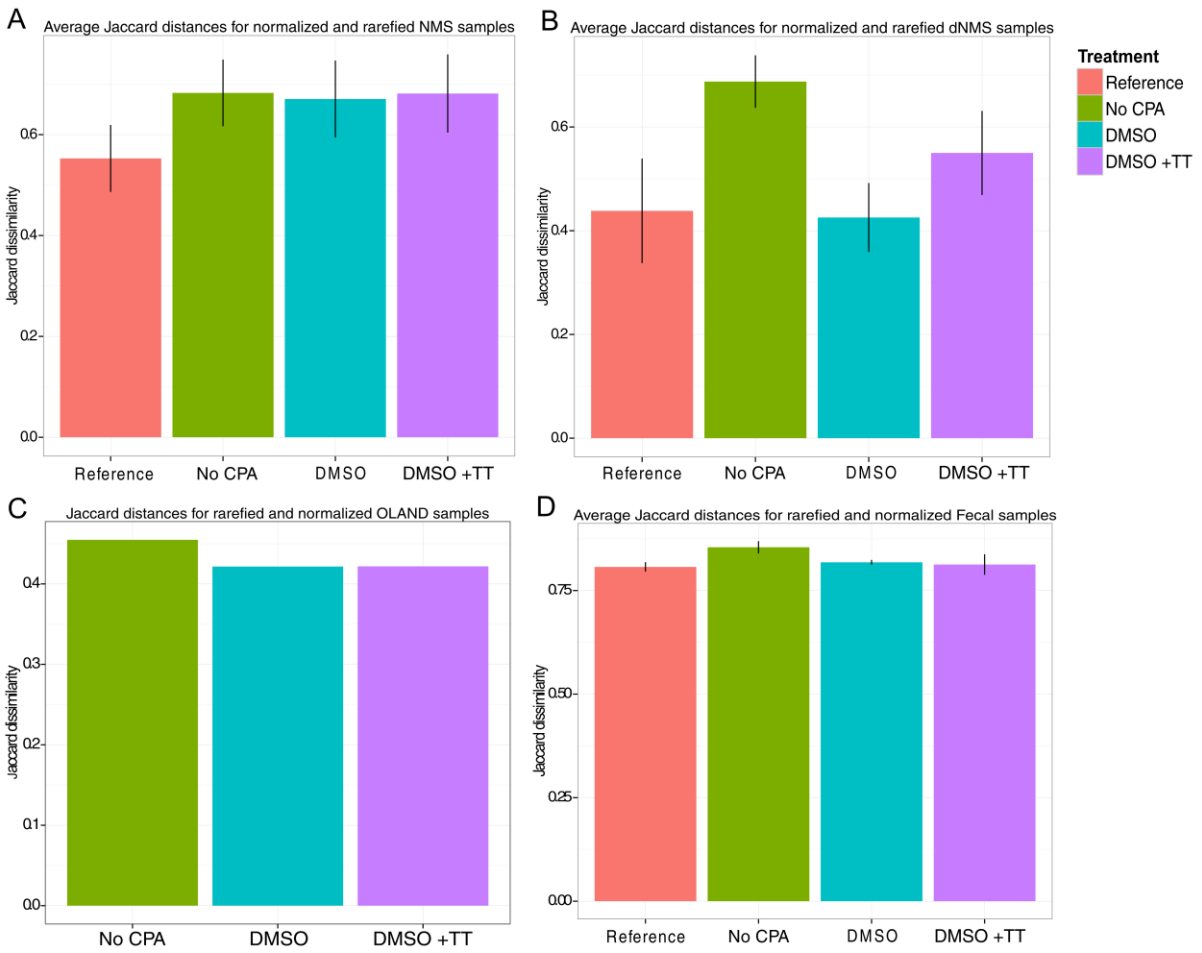

Figure 5-13. Average abundance-based Jaccard (Ružička) distances between experimental stages and conditions. *The distances are displayed for MOB biomass cultivated on NMS (A), dNMS (C), OLAND biomass (B) and fecal biomass (D). The reference represents the distance between t_0 and t_1 whilst the other bars represent the distance between t_0 and the several conditions at t_3. Error bars (for MOB and fecal samples) represent standard deviations (n=4 in A, n=6 for reference in C and 4 for the other means, n=4 in D). No reference is available for the OLAND biomass because timepoint t_1 was not assessed using Illumina.*

4. Discussion

The aim of this research was to evaluate a cryopreservation design allowing availability of a functionally and structurally reproducible inoculum for scientific and technological applications. A satisfactory recovery of specific activity of the three bacterial mixed communities studied was achieved. A critical evaluation of the community structure and differences in relative abundances or membership of the community constituents confirmed community stability, thus guaranteeing functionalities in future performance in distinct set-ups.

4.1. CPA addition enhances fast recovery of autotroph-driven consortia

Previous work on pure cultures of fastidious bacteria, such as AnAOB (Heylen, Ettwig *et al.* 2012), AerAOB (Hoefman, Pommerening-Roser *et al.* 2013), NOB (Vekeman, Hoefman *et al.* 2013) and MOB (Hoefman, Van Hoorde *et al.* 2012) has shown that these bacteria require addition of the appropriate CPA for survival during cryopreservation (Heylen, Hoefman *et al.* 2012). This was indeed confirmed with the MOB mixed culture and the AnAOB and AerAOB (with DMSO+TT) in the OLAND mixed community. Gel-entrapped AerAOB and NOB (denitrifying sludge) were previously shown to have better preservation in the absence of a CPA (Vogelsang, Gollembiewski *et al.* 1999). However neither DMSO nor DMSO+TT were evaluated for the gel-entrapped sludge. Also, in the current study, OLAND biomass was not gel entrapped but part of a RBC biofilm. In contrast to the findings on gel entrapped AerAOB, addition of a CPA (DMSO+TT) enabled AerAOB recovery comparable to the initial activity in this experiment. The increase in activity recovery on DMSO+TT and the reduced recovery on DMSO alone corroborate with earlier findings for *Nitrosomonas* spp. (Hoefman, Pommerening-Roser *et al.* 2013) for preservation at -80°C. No pre-preservation growth was executed with TT medium, which is known to enhance activity recovery of certain *Nitrosomonas* spp. (Hoefman, Pommerening-Roser *et al.* 2013). Earlier findings for AnAOB showed that addition of TT along with DMSO without any pre-incubation enhanced recovery over DMSO without TT (Heylen, Ettwig *et al.* 2012). These findings aid in elucidating the overall impact of the time point of carbon addition to the preservation medium, which remain poorly understood.

The findings for NOB corroborate with earlier findings for gel entrapped NOB, since their best activity recovery was realized without CPA, as well as with more recent findings, where nearly all NOB strains resuscitated well after cryopreservation without addition of a CPA (Vekeman, Hoefman *et al.* 2013). Furthermore, it has been shown that the optimal DMSO concentration for cryopreservation of certain NOB is 10% (v/v) whereas in the current study only 5% DMSO was evaluated (Vekeman, Hoefman *et al.* 2013).

Previous research on cryopreservation of the entire OLAND consortium at -20°C showed that AnAOB activity recovery failed (Vlaeminck, Geets *et al.* 2007). To our knowledge, the current work is the first report of a successful AnAOB activity recovery after cryopreservation of the OLAND mixed community, however previous reports of cryopreservation of both aggregated and single-cell highly enriched AnAOB cultures exist (Heylen, Ettwig *et al.* 2012).

For the fecal biomass, the SCFA production is a result of the saccharolytic metabolism of several cross-feeding heterotrophic bacteria in the community. Because of the high community diversity, the non-fastidious nature of heterotrophic bacteria and the rich nutritional background from where this mixed culture originated, the biomass seemed to be more 'robust' to cryopreservation. This was clearly demonstrated by the fact that addition of a CPA did not markedly improve activity recovery. The finding that a heterotrophic microbial consortium was not aided in fast recovery by the addition of a CPA is in contrast with earlier findings (Laurin, Labbe *et al.* 2006) for methanol-fed denitrifying biomass.

4.2. Preserving community structure

It has been established that different preservation conditions (i.e. a different CPA) influence the success of cryopreservation with a great variability among pure cultures on a species- or even strain-level (Smith and Ryan 2008). In the case of the mixed methanotrophic community, both the effects of cryopreservation on the key ecosystem drivers (the MOB) as well as the peripheral heterotrophic community (Hanson and Hanson 1996; Hrsak and Begonja 2000; Murase and Frenzel 2007; van der Ha, Vanwonterghem *et al.* 2013; Ho, de Roy *et al.* 2014) are of interest. Concerning the mixed community drivers, type I MOB (Gammaproteobacteria) and type II MOB (Alphaproteobacteria) show distinct ecophysiological features (Hanson and Hanson 1996) and have been suggested to possess different life strategies (Ho, Kerckhof *et al.* 2013). Hence, to allow a mixed methanotrophic culture to perform in a broad range of circumstances, representatives of both type I and type II MOB should be preserved during cryopreservation. Both gamma- and alphaproteobacterial MOB were recovered after cryopreservation. *Methylocystaceae* (Alphaproteobacteria) did not require addition of a CPA to maintain relative abundances in the mixed culture. As our analyses were DNA-based, it is possible that the detected alphaproteobacterial MOB are part of the microbial seed bank in the reactor (as previously demonstrated for soils (Eller, Kruger *et al.* 2005; Krause, Lüke *et al.* 2012)). Alphaproteobacterial MOB are known to have more persistent resting cells than gammaproteobacterial MOB (Whittenbury, Phillips *et al.* 1970); hence, the addition of a CPA does not influence their cryopreservation. This is in agreement with the diagnostic microarray results where the least changes in MOB diversity occurred when no CPA was added (the micro-array was only run on the NMS samples) and where *Methylocystis* sp. (strain M or related) was reduced in relative abundance when no CPA was added (Figure 5-6).

It has been shown that methanotrophs support heterotrophic bacteria by supplying the carbon-source for methanotrophic mixed culture. Little is known about the interactions between the methanotrophs and heterotrophs (van der Ha, Vanwonterghem *et al.* 2013). Nonetheless, these interactions are very specific (Hrsak and Begonja 2000; Iguchi, Yurimoto *et al.* 2011; Stock, Hoefman *et al.* 2013; van der Ha, Vanwonterghem *et al.* 2013) and allow for adaptability to a broad range of conditions (Hrsak and Begonja 2000). Because of the importance of these interactions (Hrsak and Begonja 1998; Helm, Wendlandt *et al.* 2006; Su, Xia *et al.* 2014), investigation of the total community structure before and after cryopreservation was performed within the scope of this study. Although, a differential impact was seen on 21% of the MOB community, this was not linked to phylogeny, even at the genus level. For instance, most OTUs classified as *Methylotenera* occurred in every experimental condition, while others were enriched after cryopreservation, and even others required addition of a CPA for cryopreservation on dNMS. Furthermore, OTUs belonging to *Devosia*, *Methylobacillus*, *Rubrivivax* required a CPA for cryopreservation on NMS. All manipulations were performed at 4 °C to avoid DMSO cytotoxicity and, while some OTUs did not survive when DMSO alone was used as a CPA, no single taxonomic group was found to be more sensitive than others.

The autotrophic drivers in the OLAND community consist of relatively small part of the total community, accounting for about 43-61% of the total bacteria in a RBC biofilm and 58-74% in a granule (Egli, Bosshard *et al.* 2003; Vlaeminck, Terada *et al.* 2010). For an OLAND RBC biofilm, AerAOB, AnAOB and NOB were present at 10-28%, 33% and <5% of total cells, respectively, as determined by FISH (Vlaeminck, Terada *et al.* 2010). In the current study, 6% of the total community could be classified as AerAOB, 10% as AnAOB and 0.1% as NOB. The comparatively low percentage of AnAOB might be due to underrepresentation of the *Planctomycetes* phylum in the current 16S rRNA gene sequence databases (Chiellini, Munz *et al.* 2013) or, until recently, the lack of a proper PCR protocol for the phylum (Cayrou, Sambe *et al.* 2013). Interestingly, even though NOB have a higher relative abundance with DMSO+TT after cryopreservation, their activity recovery was the lowest. This might result from a competition for nitrite with AnAOB that have the best activity recovery when DMSO+TT was used as a CPA. The increased activity recovery of AnAOB could result from the effect of DMSO on the phospholipid bilayer (Fuller 2004) of intracytoplasmatic membranes which contain the key enzymes for ammonium oxidation (Hoefman, Pommerening-Roser *et al.* 2013). Besides the autotrophic functional community members, filamentous bacteria from the phylum *Bacteriodetes* and bacteria belonging to the

phylum *Actinobacteria* were described (Egli, Bosshard *et al.* 2003) in the OLAND biomass. However, not much is known about the role of the peripheral heterotrophic community in the OLAND community. The current OLAND community shows presence of both *Actinobacteria* and *Bacteroidetes*. Only one genus required CPA addition for every representative to be cryopreserved: *Leptonema*. Some, but not all, unclassified *Rhizobiales* required the addition of TT to DMSO whereas this addition was a prerequisite for the recovery of the sole representative of the *Bdellovibrionales* order. All representatives of *Geosporobacter thermotalea, Thauera, Anaerovorax, Methylomonas, Peptinophilus, Bacteroides* and *Desulfovibrio* were enriched after cryopreservation. The only representative of the *Veillonella* genus occurs only after cryopreservation with a CPA. Apart from the general peripheral heterotrophic community, peripheral methylotrophs such as MOB were also detected in OLAND biomass (Ho, Vlaeminck *et al.* 2013), and could mitigate methane emissions from the OLAND WWTP. Gammaproteobacterial MOB were detected in all conditions after cryopreservation up to 0.05% of relative abundance in the conditions where DMSO and DMSO+TT were added.

In the fecal community, many different taxonomic families were implicated in the SCFA production. Because of the high diversity and number of representatives in most taxonomic levels, no clear influence of cryopreservation on taxonomic group representation in the fecal microbiome was discerned. The only existing study on cryopreservation of vertebrate fecal biomass shows that addition of a CPA aided in recovery of the growth of bacterial cells (Waite, Deines *et al.* 2013).

Some OTUs (8% (MOB, OLAND) to 15% (Fecal community)) were not detected at t_0 but do occur at t_1 or t_3. The most probable explanation is a very low sequence count of these OTUs in the initial inoculum which might have been either processed out in OTU binning or "rarefied out" when subsampling to lowest sample sequence count.

In contrast to the investigation of individual (taxonomic) community changes, the assessment of overall community structure is a more robust approach to uncover community structure. This approach has a greater ecological and methodological relevance as it aims at quantifying the global community changes rather than relying upon classification and taxonomy. It is clear that the Jaccard dissimilarity was less when a CPA was added during cryopreservation for each of the evaluated microbial cultures. Constrained canonical correspondence analysis integrates both functionality and community structure data. This analysis supports the conclusions from the comparison of Jaccard dissimilarities.

5. Conclusion & perspectives

A cryopreservation protocol for mixed microbial cultures was evaluated over three months with three different bacterial mixed cultures. The use of DMSO + trehalose and tryptic soy broth as a CPA consistently gave the best success rate although the cryopreservative was not necessary to obtain adequate cryopreservation of fecal material. The functionality recovery in a three-month cryopreservation experiment was previously shown to be similar in longer duration experiments (6-12 months; Hoefman, Van Hoorde *et al.* (2012)).

From an ecological point of view, even with CPA addition, not all OTUs were preserved. However, no significant differences in overall community structure were found. Although a perfect preservation of community structure was not obtained, one might question the importance of a single OTU in the community structure if taxonomically related (and hence possibly functionally redundant) OTUs are still present in the community, as was observed here. However, to allow the community to perform under different circumstances and possibly even different functionalities than the ones evaluated here, as many OTUs as possible should be retained. Hence, when cryopreserving mixed microbial communities, community structure should always be verified pre- and post-freezing and benchmarked with the biological dynamics in community structure, as performed in the current experiment as well.

From a biotechnological point of view, CPA addition was necessary for fast and reproducible activity recovery. Only with the fecal material, optimization of the method is necessary. Overall a reproducible storage method was found where addition of DMSO+TT as a CPA outperforms the limited state-of-the art preservation techniques for mixed microbial cultures. As adequate activity recovery can be obtained without introduction of an extended lag phase, undoubtedly this methodology will boost the use of mixed cultures in biotechnological applications.

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CHAPTER

6

OVERALL DISCUSSION AND CONCLUSION

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

1. General research outcomes and positioning of this research.

1.1. Positioning of this research

Methane (CH_4) is a greenhouse gas (GHG) with important contributions to climate change (Ciais, Sabine *et al.* 2014): CH_4 could contribute up to 20% of the global warming effect (Yusuf, Noor *et al.* 2012). The atmospheric CH_4 mixing ratio continues to increase in magnitude (Nisbet, Dlugokencky *et al.* 2014) which can be attributed to the disruption of the atmospheric CH_4 budget from anthropogenic activity since pre-industrial times (Kirschke, Bousquet *et al.* 2013; Ciais, Sabine *et al.* 2014). However, due to the relatively short atmospheric lifetime of CH_4, the CH_4 budget could be balanced by a relatively modest decrease of 6% in global CH_4 emissions (Jardine, Boardman *et al.* 2004). Interestingly, in both natural and engineered systems methane oxidizing bacteria (MOB) are found to attenuate CH_4 release to the atmosphere, making them viable target organisms to achieve mitigation of anthropogenic CH_4 emissions.

While axenic MOB isolates are interesting organisms for the study of biological methane oxidation, increasing evidence points out that methanotrophic *interactomes* (consisting out of MOB and non-MOB partners) may outperform the axenic cultures and be the actual drivers of biological methane oxidation rather than a single MOB (Stock, Hoefman *et al.* 2013; van der Ha, Vanwonterghem *et al.* 2013; Ho, de Roy *et al.* 2014; Oshkin, Beck *et al.* 2015).

The goal of this dissertation was to investigate the composition and possible interactions in methanotrophic *interactomes*. Ultimately, an increased understanding of these essential interactions for biological CH_4 oxidation may lead to an improved microbial resource

management (MRM, Read, Marzorati *et al.* (2011)) and hence biotechnological applications of the MOB:non-MOB *interactome* (Jagmann and Philipp 2014; Pandhal and Noirel 2014; Verstraete 2015).

1.2. Main research outcomes

The main subject of this dissertation are methanotrophic *interactomes* between methane oxidizing bacteria and non-MOB partners.

In a first part of the dissertation, a lab-enriched MOB *interactome* was dissected in a top-down approach by means of stable isotope probing at shorter timescales than previously reported. In Chapter 2 it was observed that $^{13}CH_4$-derived carbon is differentially distributed among *interactome* partners through time. Therefore, it was hypothesized that the most intricately associated non-MOB partners would be the first to assimilate $^{13}CH_4$-C and could hence be considered "primary consumers" whereas organisms labelled later in the time-course SIP experiment are more loosely associated with the MOB and may be "secondary consumers" of organic carbon derived from both the MOB and the primary consumers. This finding could become of significance for *interactome* MRM, if the biotechnological application of secondary partners is the ultimate goal.

The experiments second part of the dissertation set out to assemble the methanotrophic *interactome de novo*, by means of two bottom-up approaches. In Chapter 3 a supervised assembly approach was used where partners for the MOB were selected based upon their initial compatibility and subsequently subjected to repeated sub-cultivation cycles. Interestingly, no adaptation of a moderately compatible partner to MOB (or vice versa) could be observed during repeated co-cultivation with a gammaproteobacterial MOB. Conversely, clear (though limited) adaptation was observed with an alphaproteobacterial MOB. Regardless of its initial compatibility with the MOB, a third partner nearly always completely obliterated the non-MOB partner in an existing MOB:non-MOB *interactome*. In Chapter 4, an unsupervised *interactome* assembly approach was employed. Here, 8 non-MOB partners had to compete for CH_4 derived carbon from either an alpha- or gammaproteobacterial MOB during repeated cycles of co-cultivation. A clear partner selection was observed where some of the persisting partners were "promiscuous" and could persist regardless of MOB type , whereas others were more specifically associated with either MOB type. Additionally, alpha- and gammaproteobacterial MOB were found to differentially route CH_4-derived carbon to the *interactome*. Finally, while repeated co-cultivation did not significantly impact the magnitude

of overall methane oxidation rates (MOR), it did appear to stabilize the biological variability in MOR as compared to axenically grown MOB. Finally, mining of the *interactome* partner's genomes revealed clues on partner selectivity such as the importance of a gene encoding for cobalamin importers. Overall, unsupervised *interactome* assembly approaches should be preferred, as they specifically restrict only non-MOB partners that can persist with the MOB and other non-MOB partners.

Finally, in a third part of this dissertation new storage methods for *interactomes* were investigated. If an optimal combination of *interactome* partners for specific biotechnological applications can be found, it is of great interest to preserve a reproducible sample of it for extended periods of time. Therefore, in Chapter 5 an optimized cryopreservation protocol for storage of mixed microbial cultures is described, which adequately preserved both community structure and functionality of a methanotrophic *interactome* (among others) for 3 months at -80°C.

1.3. Methanotrophic *interactomes* and sustainable methane cycling.

One of the main underlying motivations of the research within this thesis was to investigate how microbial partnerships can increase sustainability of methane cycling. One of the methods used to investigate this was *de novo* assembly of methanotrophic *interactomes* by combining MOB with non-MOB partners (Chapters 3 and 4). While there are indications that increased diversity enhance methane oxidation rates (Ho, de Roy *et al.* 2014) and that in a natural ecosystem a methanotrophic community (consisting of MOB and non-MOB) rather than a single MOB is responsible for methane cycling (Oshkin, Beck *et al.* 2015), which give implications that microbial partnerships make biological CH_4 oxidation more efficient and hence more sustainable, in this manuscript underlying reasons and mechanisms for these interactions were investigated. Ultimately, by an improved microbial resource management (Read, Marzorati *et al.* 2011) optimal methanotrophic *interactomes* could be assembled which perform a more sustainable biological methane mitigation and methane biofiltration (Jiang, Chen *et al.* 2010; Ganendra, Mercado-Garcia *et al.* 2015). Although a boost in MOR by adding non-MOB was not observed (Chapter 4, section 2.3), this may have been a density-dependent effect (Jeong, Cho *et al.* 2014). The (limited) adaptation of LMG26262 to its fixed partner in Chapter 3, section 3.2 could indicate that sustainable CH_4 oxidation can be evolved using synthetic ecosystems in the lab. Nevertheless, in Chapter 4, section 2.3 a reduced variability in MOR was observed, which is essential for application of the *interactome* in

sustainable CH_4 biofiltration. Furthermore, *interactomes* could be assembled to contain non-MOB partners able to remove other unwanted compounds in the CH_4 containing waste-stream (such as landfill or coal gas, Jiang, Chen *et al.* (2010)). Given the requirement for oxygen (Equation 1-2), interactions with algae can also increase economic sustainability of CH_4 oxidation (van der Ha, Nachtergaele *et al.* 2012). Ultimately, looking at the results of Chapters 2, 3 and 4 specific synthetic *interactomes* could be assembled to even further increase economic sustainability of CH_4 cycling, if non-MOB partners can be microbes manufacturing specific products with high economic value. Given the results of Chapters 3 and 4 the use of alphaproteobacterial rather than gammaproteobacterial MOB may be essential to develop such *interactomes*.

2. The mode of interaction between MOB and non-MOB partners

Overall, this dissertation mainly investigated co-occurrence and differential abundance among MOB and their non-MOB partners. While these co-occurrence patterns are interesting and genomic information can infer clues for possible underlying selective mechanisms (Chapter 4), they do not conclusively show which molecules are being exchanged. However, based upon time-resolved co-occurrence as in Chapter 2 it is interesting to observe the most intricate association between MOB and methylotrophic non-MOB of the *Methylococcaceae* and *Methylophilaceae* families, which confirms earlier reports of frequent co-occurrence of these two groups (He, Wooller *et al.* 2012; Hernandez, Beck *et al.* 2015; Oshkin, Beck *et al.* 2015). The tight association between MOB and methylotrophic non-MOB could be determined by the sensitivity of MOB to CH_3OH (section 2.2.1 of Chapter 1). While sensitive MOB may be relieved from CH_3OH stress by associations with methylotrophic non-MOB, they could also be relieved by CH_3OH-insenstive MOB. However, in the last case a competition for the available CH_4 may render this interaction less favourable. We did not observe a clear link between initial partner compatibility of non-MOB partner strains and described methylotrophic metabolism in their respective genera (Chapter 3), although generally the highly compatible strains had a higher representation of methylotrophs in their genera. Furthermore, genome mining did not show clear distinctions in methylotrophic metabolism for persisting or non-persisting non-MOB partners in Chapter 4, where genes for sugar metabolism and genes involved in vitamin B12 transport were reported to be distinctive. However, it is of importance to consider that the metabolism of C1 compounds by non-MOB partners doesn't necessarily need to be *sensu stricto* methylotrophic: rather than anabolic and

catabolic coupling of C1 metabolism (i.e. as carbon- and energy source), catabolic demethylation and reduction to CO_2 to generate reducing equivalents is an alternative physiological mode of C1 metabolism. In distinction to obligate and facultative methylotrophs, we will further refer to these organisms as methylovores (Sun, Steindler *et al.* 2011). In this way we hypothesize that the MOB could supply electron donors to e.g. the SAR11 pelagic Alphaproteobacteria (Sun, Steindler *et al.* 2011) and *Thermomonas* spp. (Wei, Ye *et al.* 2015) or both electron donor (C1 compounds) and carbon source (CO_2) to autotrophic methylovores such as *Methylonatrum kenyense* (Sorokin, Trotsenko *et al.* 2007). Furthermore, (facultative) methylotrophic metabolism may be more wide-spread than previously thought, with new methylotrophs (and methylovores) being isolated over time (Boden, Thomas *et al.* 2008; Chistoserdova, Kalyuzhnaya *et al.* 2009; Chistoserdova 2011; Hung, Wade *et al.* 2011). Hence, as knowledge of methylotrophic metabolism increases, new enzymes involved pop up. A nice example is XoxF, a methanol dehydrogenase (MDH) homolog whose function was initially unknown (Chistoserdova 2011), XoxF was found to require rare-earth elements (REE) of the lanthanide series which precluded clarification of its functionality (Keltjens, Pol *et al.* 2014). Nevertheless, REE-dependent XoxF-MDH is presumed to have a higher catalytic activity than conventional MxaFI-type MDH (Wu, Wessels *et al.* 2015; Chu and Lidstrom 2016). Furthermore, XoxF-type MDHs are quite wide-spread among bacteria in the environment, leading to a higher diversity of methylotrophic organisms than could be expected based upon conventional MxaFI-MDHs (Minamisawa, Imaizumi-Anraku *et al.* 2016). Therefore, it is not unthinkable that yet undiscovered mechanisms of methylotrophy (or methylovory) exist, precluding a clear view on the mechanism of the MOB:non-MOB reciprocal selectivity observed in Chapters 3 and 4.

Interestingly, the formation and excretion of soluble EPS/soluble sugars by MOB (van der Ha 2013; Khmelenina, Rozova *et al.* 2015) may be another selective mode of interaction between MOB and non-MOB given the findings from genome mining in Chapter 3. Additionally, synthesis of extracellular polymeric substances (EPS) could be performed by the MOB in, which were generally supplied with excess CH_4, resulting in the excretion of sugar-based polymers as a way to shunt toxic formaldehyde out of the MOB cells (Huber-Humer, Gebert *et al.* 2008) which additionally reduces oxygen diffusion (Hilger, Cranford *et al.* 2000), de facto creating a micro-aerobic environment for the MOB (which can hence differ from the oxygenated bulk environment). It could be hypothesized that in these hypoxic EPS micro-environments CH_4 metabolism may switch from respiratory to fermentative (Kalyuzhnaya, Yang *et al.* 2013) thereby releasing interesting metabolites such as organic acids and ethanols

to non-MOB partners. Finally, the role of cobalamin in MOB:non-MOB interactions requires further attention (Chapter 3, Iguchi, Yurimoto *et al.* (2011)).

3. Alpha- and gammaproteobacterial MOB differentially mediate interactions with their non-MOB partners

From the results of Chapters 3 and 4 it is clear that the alpha- and gammaproteobacterial MOB employed in this dissertation (*Methylosinus* sp. LMG 26262 and *Methylomonas methanica* NCIMB 11130^T respectively) had a differential impact and selectiveness towards their non-MOB partners. Whether this is an effect specifically associated to these strains, or rather typical characteristics of their respective phylogenetic groups still needs to be determined. However, generally our conclusions were in accordance with the presumed life strategies of these groups (Ho, Kerckhof *et al.* 2013): the highly competitive gammaproteobacterial *M. methanica* was less prone to mutually beneficial associations than the stress-tolerator alphaproteobacterial *Methylosinus*. Typically observed beneficial mixing ratios of MOB:non-MOB tend to support this hypothesis (Iguchi, Yurimoto *et al.* 2011; Jeong, Cho *et al.* 2014).

Rather than stimulatory impact of non-MOB partners on MOR (Ho, de Roy *et al.* 2014; Jeong, Cho *et al.* 2014) stabilization of the variability of the MOR was observed in Chapter 4. While no clear impact on headspace CO_2 concentrations could be observed, a relatively greater amount of respiration to CO_2 (by the additional respiration caused by non-MOB partners) may have been a stabilizing effect on MOR, certainly for alphaproteobacterial MOB which require CO_2 for biomass assimilation through the serine cycle (Acha, Alba *et al.* 2002; Yang, Matsen *et al.* 2013).

Further research is required to elucidate if and how the difference in CH_4-derived carbon assimilation of alpha- and gammaproteobacterial MOB influences interactions with non-MOB partners. To do so, an extension of the experiments performed in Chapters 3 and 4 with a greater number of different and related proteobacterial MOB would be an interesting approach to determine if indeed these two groups of MOB have differential interactions with their non-MOB partners. To elucidate the underlying mechanisms however, the construction of a multi-species metabolic model could clarify exchange fluxes through e.g. dynamic flux balance analysis (dFBA, (Zhuang, Izallalen *et al.* 2011; Khandelwal, Olivier *et al.* 2013)). For a relatively simple 1:1 MOB:non-MOB *interactome* such a multi-species model could be readily formulated and parameterized. Tuning and validation of these analyses will require

high-throuhput time-resolved metabolomics (Lee, Gianchandani *et al.* 2006), which could be enhanced by using $^{13}CH_4$ as a substrate. Furthermore, selective knock-outs could arrest the pathways of interest or transport molecules. The increasing development of genetic tools for methanotrophs (Ye, Yao *et al.* 2007; Smith and Murrell 2011; Crombie and Murrell 2014; Puri, Owen *et al.* 2015) can be applied to create mutants that can validate the hypotheses developed from community-wide metabolic models.

4. Spatial heterogeneity, micro-niches and representativeness of the completely mixed batch incubations

All experiments in this thesis were executed in batch incubations on a rotary shaker at 120 rpm. This condition, just like the use of NMS rather than dNMS and 20% (v/v) CH_4 in air in the headspace rather than lower CH_4 or O_2 concentration, was chosen to have no limiting factors which could preclude MOB growth. The shaking creates a turbulent flow of the medium inside the batch incubation bottles, which increases mixing of the liquid with the headspace and hence increases the gas-liquid transfer rates. Given the relatively low solubility of CH_4 and O_2 in NMS at 21-28°C these conditions were believed to be necessary to stimulate MOB growth. Both of the MOB were cultured lab "workhorse"-organisms (certainly NCIMB 11130^T), which were isolated in and accustomed to these cultivation conditions. However, generally, completely mixed conditions rarely occur in nature where MOB reside within sediments and soils at the oxic/anoxic interface or at the oxycline in stratified lakes (Hanson and Hanson 1996; Kolb 2009; Bowman 2014; Knief 2015). This also became apparent also when enrichment of MOB from e.g. North Sea sediment was rendered near impossible under non-static incubation while presence of sediment particles during enrichment was found to enhance MOR (Vekeman 2016). Hence, the great bias in the availability of MOB isolates as opposed to *pmoA* sequences retrieved by metagenomics approaches (Knief 2015) may reside in the fact that isolation and enrichment protocols are not representative for the natural conditions. For instance, the recent discovery of new spiral-shaped methane oxidizers required micro-oxic conditions and they were shown to exhibit chemotaxis towards CH_4 in a semi-solid medium (Danilova, Suzina *et al.* 2016), illustrating the impact of cultivation conditions on the discovery of new MOB. However, how the lack of spatial heterogeneity potentially impacts research into MOB:non-MOB interactions still remains an open question.

Regardless of the spatial heterogeneity, a tight physical association among MOB and non-MOB partners may be required if direct metabolic exchange occurs. This could be illustrated

for instance by non-motile *Methylobacterium*-like methylotrophs "horse-riding" the mobile spiriloform MOB by a tight attachment to their cells (Danilova, Suzina *et al.* 2016). Other forms of tight association between MOB and non-MOB occur in biofilms or floc-like aggregates, which would create micro-oxic niches by diffusion barriers. In this case, fermentative methane metabolism (Kalyuzhnaya, Yang *et al.* 2013) could supply interesting metabolites for the non-MOB to proliferate within the methanotrophic *interactome*.

On the other hand, if the exchanged metabolites between MOB and their non-MOB partners are gaseous (CO_2, O_2) or volatile, a tight association might not be required. Interesting in this respect are experiments and observations on the interaction between MOB and (micro)-algae, for instance photosynthetic algae creating a new niche for the MOB in the water column of a seasonally stratified anoxic lake (Milucka, Kirf *et al.* 2015) in this case, gammaproteobacterial MOB were free-living in the water column and benefiting from the photosynthetically produced oxygen. This was also exemplified by a two-stage separate algae and MOB reactor system with a shared headspace (gas-bridge) converting biogas to lipids and PHB, however in a one-stage system the MOB and algae did form tight associations into bioflocs (*methalgae*, van der Ha, Nachtergaele *et al.* (2012)).

Ideally, future experiments should evaluate how the degree of spatial heterogeneity affects the extent of MOB:non-MOB partnerships. One approach may be to use CH_4/O_2 counter-gradient cultivation systems (Bussmann, Rahalkar *et al.* 2006; Krause, Lüke *et al.* 2012) with differing agarose concentrations in the semi-solid columns (with or without added sterile glass beads simulating soil or sediment sand particles (Danilova, Suzina *et al.* 2016)). Based upon clone libraries of the 16S rRNA gene Bussmann, Rahalkar *et al.* (2006) already observed a methanotrophic *interactome* containing Flavobacteria and Betaproteobacteria in association with to a gammaproteobacterial MOB with such a setup using 0.2% (w/w) agarose. Alternatively, incompletely mixed chemostat setups with baffles (intermittent or low rate stirring) could create micro-aerobic niches and wall growth (Wilkinson and Hamer 1974), certainly if operated in a retentostat mode (i.e. with biomass retention). Also here, the extent of homogeneity within the cultivation system can be regulated (even more easily than in gradient columns) and hence serve as a model parameter to explicit spatio-temporal modelling. This kind of modelling only becomes relevant under incomplete mixing and spatial heterogeneity, otherwise the system can just be described using ordinary (ODE) and partial differential equations (PDE). However, certainly in terms of fully parameterized individual-based models (IBM), mechanistic explanations and hypotheses for collaboration

and competition within the (methanotrophic) *interactome* could be found (Larsen, Hamada *et al.* 2012; Esser, Leveau *et al.* 2015).

Nevertheless, despite their ecological relevance, *interactome* cultivation with a matrix or gradient system to introduce spatial heterogeneity may not be desirable for high-rate biotechnological product manufacturing applications where continuously stirred tank reactors (CSTR) are commonplace (Helm, Wendlandt *et al.* 2006). On the other hand different, more stratified, reactor designs have been applied for e.g. PHB production by MOB in a fluidized bed reactor (Pfluger, Wu *et al.* 2011; Pieja, Sundstrom *et al.* 2012) or SCP production with the U-loop reactor system (Prado-Rubio, Jørgensen *et al.* 2010). Additionally, the use of membrane biofilm reactors (MBfr) or membrane-aerated (with a CH_4/O_2 mixture) biofilm reactors (MABr) may be advantageous for biofilter-like applications (Hamer 2010; Modin, Fukushi *et al.* 2010; Sun, Dong *et al.* 2013). Miniaturization and multiplexing on a bench/lab-scale of these reactor types could allow for a controlled heterogeneous continuous reactor system to inoculate with (synthetic) *interactomes* and assess MOB:non-MOB interactions.

Chapter 7

Perspectives for future research

CHAPTER 7

PERSPECTIVES FOR FUTURE RESEARCH

1. Synthetic ecosystems

Given the (extreme) niche differentiation observed at intergeneric, interspecific and even inter-strain levels of MOB (Hoefman, van der Ha *et al.* 2014) as well as differentiation among other closely related non-methanotrophic methylotrophs (Jimenez-Infante, Ngugi *et al.* 2016) and non-methylotrophs (Johnson, Zinser *et al.* 2006) to enable distinct niche occupation, synthetic ecology approaches, as the ones employed in this dissertation can address hypotheses about the methanotrophic *interactome* where the standard molecular toolbox for microbial community dissection falls short. The synthetic ecosystems toolbox can additionally offer potential for industrial biotechnology: with a lower species diversity, biotechnological processes using a synthetic ecosystem possess higher reproducibility and controllability compared to natural mixed communities (De Roy, Marzorati *et al.* 2014). Synthetic communities also present a solution for typical problems encountered with pure cultures. Firstly, they can be designed to have a broader metabolic flexibility and catalyse different processes more efficiently by cooperative combination of metabolic pathways and enzymatic systems of different organisms. Secondly, the stability of synthetic communities is a consequence of both chemical and cellular interactions between the community members and is typically higher than that of axenic cultures (Jagmann and Philipp 2014; Pandhal and Noirel 2014). Here I would like to advocate that future research on the methanotrophic *interactome* and its possible applications should greatly benefit from applying the engineering paradigm to synthetic methanotrophic *interactomes* (Figure 7-1). Additionally, the availability of the full genome of each constituent strain of a synthetic ecosystem (as in Chapter 4) is advantageous for other 'omics' applications to further unravel how gene-expression (metatranscriptomics) and translation to proteins (metaproteomics) is influenced by repeated co-cultivation and

possible adaptation of MOB and their partners in a methanotrophic *interactome*. Finally, defined synthetic ecosystems allow for proper modeling (e.g. by dynamic flux balance analyses, dFBA) and hence manipulation of the methanotrophic *interactome* (Larsen, Hamada *et al.* 2012; Jagmann and Philipp 2014; Widder, Allen *et al.* 2016).

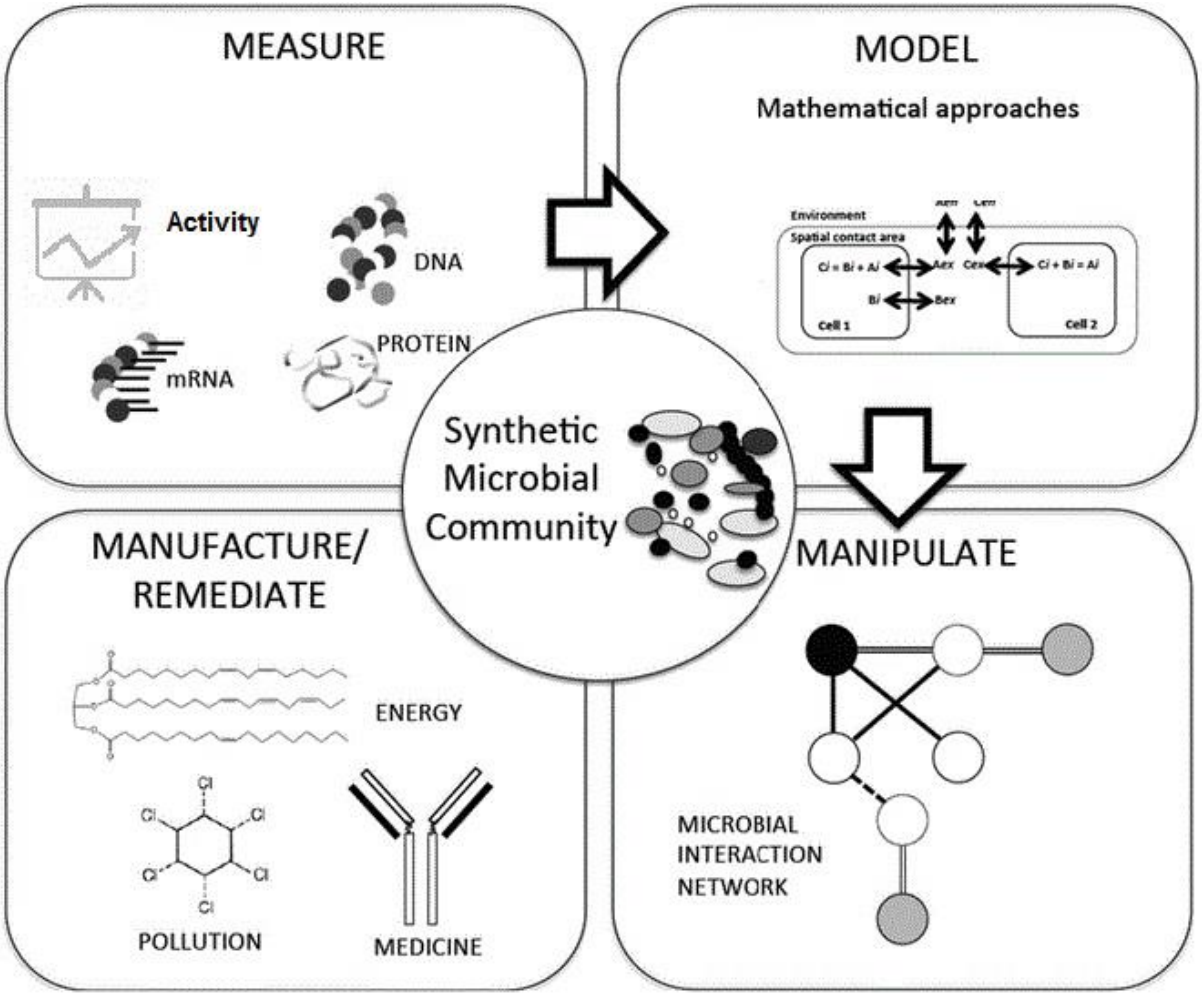

Figure 7-1. The engineering paradigm applied to artificial microbial communities, applied to methanotrophic interactomes (Pandhal and Noirel 2014). *Measure: generate quantitative data about microbial adaptation and interaction mechanisms including sequencing and measuring DNA (genomics and metagenomics), mRNA (transcriptomics and metatranscriptomics) and proteins (proteomics and metaproteomics). Model: the measured data is integrated and then interpreted by building predictive models. Manipulate: microbial communities are designed based on models with specific outcomes in mind. For example, maximising growth of a specific species and minimising the risk of contamination. The circles in the figure represent microbial species and the lines their specific interactions. The black circle represents the producer(the MOB) and hence it requires the maximum metabolic functional capacity in this system. For example, two (non-MOB) bacterial species (grey circles) are introduced to increase growth and productivity of the producer through controlling competitors of the producer (white circles). A steady state is achieved with presence of all seven species in this hypothetical scenario. Manufacture/remediate: manufacture added value compounds and mitigate methane emissions.*

The assembly of synthetic methanotrophic ecosystems (in e.g. microcosm communities, Foster and Bell (2012)) could rely on MOB:non-MOB partners which are hypothesized to closely cooperate in natural ecosystems (Oshkin, Beck *et al.* 2015). Close cooperation could drive co-occurrence of MOB and their specific non-MOB partners (e.g: Chapter 2 and section 3.2 of Chapter 1 and more generally Fiegna, Moreno-Letelier *et al.* (2015) as well as Zelezniak, Andrejev *et al.* (2015), which may evolve to a co-dependent relationship (Morris, Lenski *et al.* 2012). Hence, co-occurring and possibly co-dependent non-MOB partners could be prerequisites for MOB growth or at least stimulate it (Iguchi, Yurimoto *et al.* 2011). High-throughput isolation techniques could be applied to obtain libraries of MOB and non-MOB which are known to be adapted to one another (Chapter 2). Alternatively, assemblies of co-cultures without prior co-evolution were shown to stimulate growth (Stock, Hoefman *et al.* 2013) and MOR (Ho, de Roy *et al.* 2014), hence synergetic interactions may not be limited to co-evolved MOB:non-MOB *interactomes*. Non co-evolved partners can have the advantage that they can be selected on characteristics such as complete genome availability or differing morphology to facilitate downstream analyses of the *interactomes*, as indicated above. Assembly of the methanotrophic *interactomes* should be performed at differing evenness levels between MOB and non-MOB to account for density-dependent effects (Jeong, Cho *et al.* 2014). Furthermore, it may be relevant to repeatedly introduce a partner into the methanotrophic *interactome* to allow it to outcompete other partners in a temporal assembly strategy.

2. Continuous culture

In Chapter 3, we could not properly assess the hypothesis of adaptation due to partner loss after repeated batch cycles of co-cultivation. This could have been avoided by means of a continuous co-culture system, where only interacting partners which can co-exist at a given dilution rate will persist. Hence, in future experiments a continuous cultivation system should be employed to ensure persistence of the partner (Namsaraev and Zavarzin 1972; Wilkinson, Topiwala *et al.* 1974; Lamb and Garver 1980). By modulating the chemostat parameters such as the dilution rate and possibly biomass retention, co-cultures can be selected for specific growth rates (μ). Addtionally, high throughput screening could be performed in cheap, custom built multiplexed chemostat arrays (Miller, Befort *et al.* 2013) or micro- and miniscale bioreactors (Lattermann and Büchs 2015), which have the additional advantage of an increased volumetric mass transfer (k_La) and maximum oxygen transfer capacity (OTR_{max}),

which would be highly advantageous given the gaseous form of the CH_4/O_2 substrate for aerobic methane oxidation.

Additionally, chemostat-mode cultivation of MOB or non-MOB partners could supply *interactome* research with a continuous and reproducible stationary phase spent broth of either to elucidate if these cell-free supernatants could of MOB could stimulate non-MOB or vice versa. Additionally, a continuous flow of retentostat-mode (Tappe, Tomaschewski *et al.* 1996) biomass-free effluent which may contain stimulatory metabolites at selected growth rates. Continuous reactors could supply enough effluent for thorough biochemical characterisation of metabolites exchanged in co-cultures. Therefore, I advocate that future methanotrophic *interactome* research should be performed in continuous rather than batch cultivation systems.

3. Alternative approaches to isolation of co-dependent microbes.

Emergent properties may arise when microorganisms interact (e.g. interaction-induced production of metabolites: Watrous, Roach *et al.* (2012); Tyc, van den Berg *et al.* (2014); Abrudan, Smakman *et al.* (2015)) which could explain why the metabolites exchanged between MOB and their non-MOB partners have not been conclusively pinpointed so far based metabolome of axenic cultures of the partners. Specific systems developed for isolation of microbes which require partner microbiota have been developed based on techniques such as diffusion chambers and "microbial traps" as well as the isolation chip, consisting of miniaturized diffusion chambers (Kaeberlein, Lewis *et al.* 2002; Nichols, Lewis *et al.* 2008; D'Onofrio, Crawford *et al.* 2010; Epstein 2013). Hence, as a future research direction for better understanding of methanotrophic *interactomes* the use of diffusion chamber experiments (Figure 7-2) could elucidate if metabolite exchange over a semi-permeable membrane can stimulate growth of either the MOB, the non-MOB partner or both.

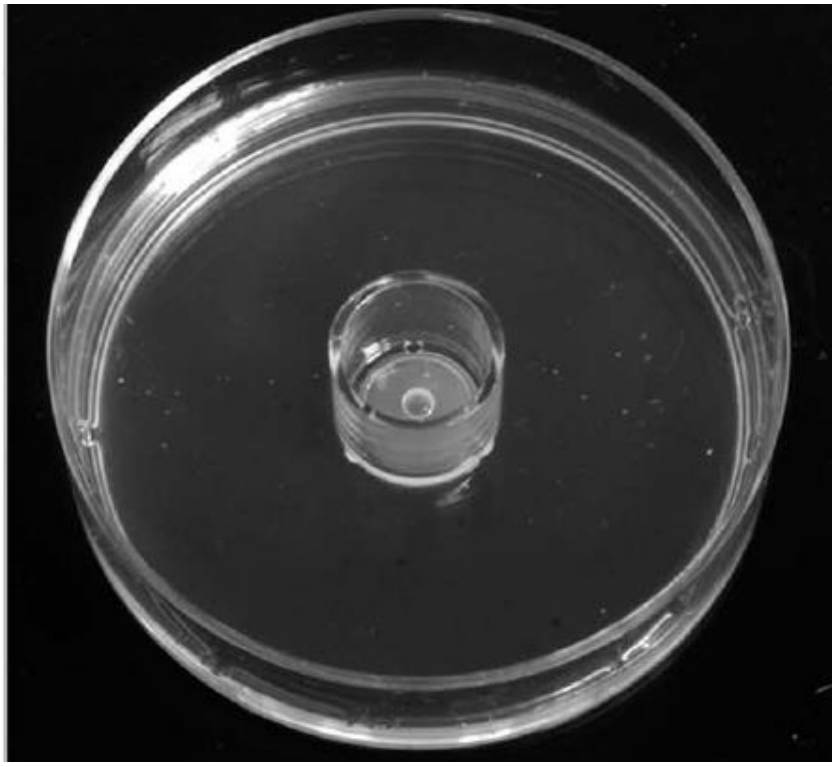

Figure 7-2. General view of a two-compartment chamber for co-cultivation of co-dependent strains. *A tissue culture insert with a porous (0.02-µm) bottom is placed on the surface of a petri dish, providing the means to grow two pure microbial cultures in chemical contact with each other. The tissue culture inserts could contain non-MOB partners, inserted on MOB-inoculated plates and incubated under CH_4 atmospheres, to assess stimulatory effects of MOB on non-MOB partners or vice-versa.*

Alternatively, in Chapter 2, $^{13}CH_4$ stable isotope probing with a high time resolution was used to identify the most intricate partners involved with the assimilation of $^{13}CH_4$ derived carbon. Selective isolation of these organisms from the active and highly enriched methanotrophic *interactome* could allow for re-assembly in a simplified synthetic ecosystem to assess hypotheses (Figure 1-10). Given the reduced cost for whole genome sequencing, the genomes of axenic isolates could be easily generated and employed in community-wide metabolic models (Chapter 6, Section 3). However, purification of these partners could prove to be challenging and possibly more advanced methods could be required. As combination of SIP with confocal Raman (and Raman-FISH) spectroscopy (Huang, Stoecker *et al.* 2007; Wagner 2009; Muhamadali, Chisanga *et al.* 2015; Wang, Huang *et al.* 2016) could be highly advantageous to track the $^{13}CH_4$ flow and cell sorting based upon ^{13}C incorporation (as recently demonstrated for heavy water with optical tweezers (Berry, Mader *et al.* 2015)). With the recent advances in Raman-Activated cell sorting (Zhang, Ren *et al.* 2015), non-destructive detection and sorting with high subsequent cell viability could be used to separate labelled

partners from the methanotrophic *interactome* into single cells which could subsequently be cultivated in e.g. spent broth of axenically grown MOB (preferably the driving MOB from the *interactome*, directly isolated from it), if growth on general media is not possible.

4. Reversing the roles: unsupervised partner selection from the perspective of the non-MOB.

While supervised *interactome* assembly was not very successful (Chapter 3), in an unsupervised competition of the non-MOB for MOB CH_4-derived carbon, clear partner selection patterns were observed (Chapter 4). While I suggested above to expand the supervised and unsupervised methanotrophic community assemblies to a wider range of methanotrophs I would additionally like to propose an experiment in which alpha- and gammaproteobacterial MOB could compete for a single (or multiple) partners in an unsupervised way. Here, the MOB do not co-depend on the partner for growth and energy though MOB which are stimulated more strongly in specific partnerships with the non-MOB will be able to prevail after repeated cycles of co-cultivation. In this setting, repeated co-cultivation should be performed for an extended period of time, possibly aided by supplying the culture with an initial amount of organic carbon to sustain the non-MOB (e.g. 1% TSB), which would wash-out with repeated sub-cultivation or dilution in continuous culture. In a preliminary experiment using both NCIMB 11130^T and LMG26262 separately with the gfp-tagged *Pseudomonas putida* SM1699 it was observed that this 1% TSB addition greatly influenced the abundance of *P. putida* after one week of co-cultivation (unpublished data), though no repeated cultivation or competition among the MOB for the *P. putida* was evaluated.

Abstract

ABSTRACT

Methane (CH_4) is an important greenhouse gas, and the majority (ca. 60%) of its emission originates from anthropogenic sources. Methane oxidizing bacteria (MOB) are characterized by their unique ability to use CH_4 as a sole carbon and energy source. Recently, accumulating evidence demonstrated that methane oxidation is stimulated when MOB are interacting with non-methanotrophic microbes. These interactions can be very specific, although it is not yet entirely elucidated what the determining factors to a successful partnership are. Hence, it seems that a methanotrophic *interactome* is required for effective biological aerobic methane oxidation, rather than individual obligate methanotrophic bacteria.

In this dissertation, we focused on bacterial interactions between MOB and non-MOB and try to assess what are the determinants for non-MOB partner selection in the methanotrophic *interactome*. We focus specifically on representatives of alpha- and gammaproteobacterial MOB, which are known to exhibit differential functional traits which can in turn be conceptualized as distinct life strategies. The impact of these differences in MOB life strategies on interactions with non-MOB has not been studied. An increased insight in the co-existence and the extent of co-dependence of MOB and non-MOB partners is required to fully understand the essential ecosystem service of biological methane oxidation.

In PART 1 (TOP-DOWN APPROACH), we dissected a methanotrophic enrichment culture (optimal methanotrophic *interactome*) by means of time-resolved stable isotope probing combined with 454-pyrosequencing of the 16S rRNA genes to assess which *interactome* partners are most intricately involved in the assimilation of $^{13}CH_4$-derived carbon. In PART 2 (BOTTOM-UP APPROACH), we artificially assembled an optimal methanotrophic interactome by piecing together multiple non-MOB strains with MOB (co-cultivation). Firstly, we supervised the selection of MOB for non-MOB partners by establishing compatibility and matching selected MOB with non-MOB partners. Microbial adaptation and abundance of all partners was investigated using q-PCR. Secondly, we allowed an

unsupervised selection for non-MOB partners by the MOB and these interactions were monitored by DGGE. In PART 3 (CRYOPRESERVETION), we develop a cryopreservation protocol based on DMSO (and DMSO+TT) as a cryopretactant, which can used to adequately preserve optimal *interactomes* (preservation of key functional characteristics as well as composition) to allow possible biotechnological applications.

In PART 1, we found that $^{13}CH_4$-derived carbon is differentially distributed among *interactome* partners through time. We suggested that the most intricately associated non-MOB partners would be the first to assimilate $^{13}CH_4$-C and could hence be considered "primary consumers" of whereas organisms labeled later in the time-course SIP experiment are more loosely associated with the MOB and may be "secondary consumers" of organic carbon derived from both the MOB and the primary consumers.

In PART 2a, we could not observe adaptation of a moderately compatible partner to MOB (or vice versa) during repeated co-cultivation with a gammaproteobacterial MOB. Conversely, clear (though limited) adaptation was observed with an alphaproteobacterial MOB. Regardless of its initial compatibility with the MOB, a third partner nearly always completely obliterated the non-MOB partner in the existing MOB:non-MOB *interactome*. In PART 2b, we showed a clear partner selection where some of the persisting partners were "promiscuous" and could persist regardless of MOB type , whereas others were more specifically associated with either MOB type. Additionally, we observed that alpha- and gammaproteobacterial MOB differentially route CH_4-derived carbon to the *interactome*. Finally, while repeated co-cultivation did not significantly impact the magnitude of overall methane oxidation rates (MOR), it did appear to stabilize the biological variability in MOR as compared to acenically grown MOB.

In PART 3, the optimized cryopreservation protocol for storage of mixed microbial cultures adequately preserved both community structure and functionality of a methanotrophic *interactome* (among others) for 3 months at -80°C.

These findings may be of significance for methanotrophic *interactome* Microbial Resource Management, if biotechnological application of non-MOB partners is the ultimate goal. Overall, unsupervised synthetic *interactome* assembly approaches should be preferred, as they specifically restrict only non-MOB partners that can persist with the MOB from other non-MOB partners. If specific biotechnological applications can be found for such an optimal combination of *interactome* partners, it is of great interest to preserve a reproducible sample

of it for extended periods of time. Exploitation and engineering of these methanotrophic *interactomes* could lead to improved and sustainable mitigation and recovery of CH_4 in the form of metabolic energy or CH_4-derived carbon.

Abstract

SAMENVATTING

SAMENVATTING

Methaan (CH_4) is een belangrijk broeikasgas, en het leeuwendeel van de methaanemissies (ca. 60%) is van antropogene oorsprong. Methaanoxiderende bacteriën (MOB) vormen een fysiologische bacteriële groep met als unieke eigenschap dat ze CH_4 als enige koolstof- en energiebron kunnen gebruiken. Er wordt echter hoe langer hoe meer bewijslast gevonden dat biologische methaanverwijdering gestimuleerd wordt wanneer MOB interacties aangaan met niet-methanotrofe microbiële partners (non-MOB). Deze interacties kunnen heel specifiek zijn, maar het is nog niet volledig duidelijk wat de achterliggende mechanismen voor deze specificiteit en de daaruit volgende geslaagde methanotrofe vennootschappen zijn. Over het algemeen kunnen we echter besluiten dat in de plaats van alleenstaande (obligate) MOB op zich, methanotrofe *interactomen* vereist zijn voor een efficiënte biologische oxidatie van methaan.

Deze doctoraatsthesis focust op bacteriële interacties tussen MOB en hun non-MOB partners en probeert we na te gaan wat de bepalende factoren zijn voor de selectie van non-MOB partners in het methanotroof *interactoom*. Verder werd de nadruk gelegd bij methanotrofe vertegenwoordigers van de Alfa- en Gammaproteobacteria. Deze grote groepen van MOB hebben verschillende functionele kenmerken die conceptueel gezien kunnen worden als verschillende levensstrategieën. Het is tot dusver onbekend hoe deze verschillende levensstrategieën interacties tussen MOB en hun non-MOB partners beïnvloeden. Om een beter inzicht te krijgen in de essentiële ecosysteemfunctie die biologische methaanoxideerders verwezenlijken was het nodig om meer inzicht te verwerven over welke MOB en non-MOB samen voorkomen en hoe deze onderling van elkaar afhankelijk zijn.

In het eerste deel (top-down benadering) werd een bestaande methanotrofe aanrijkingscultuur (en dus een optimaal methanotroof *interactoom*) uiteengepuzzeld door 454-pyrosequencing van 16S rRNA-genen te combineren met stabiele isotoop probing met een hoge tijdsresolutie. Op die manier werden net die partners in kaart gebracht die het sterkst verwikkeld waren met groei op koolstof afkomstig van $^{13}CH_4$. Vervolgens, in deel 2 (bottom-up benadering) werden

kunstmatig optimale methanotrofe *interactomen* samengesteld door non-MOB stammen te koppelen aan MOB (co-cultivering). Eerst probeerden werd een gecoördineerde benadering gebruikt waarbij initiële compatibiliteit tussen MOB en non-MOB op basis van qPCR gebruikt werd om koppels tussen MOB en non-MOB te maken. Mogelijke microbiële adaptatie en abundantie van alle partners in het *interactoom* werden opgevolgd met qPCR. In een tweede, ongecoördineerde, benadering kon de MOB zelf een selectie maken tussen verschillende non-MOB partners. Deze interacties werden opgevolgd door middel van DGGE. In deel 3 (cryopreservatie) werd een cryopreservatieprotocol op basis van DMSO (en DMSO+TT) als cryoprotectans ontwikkeld. Dit protocol kan gebruikt worden om optimale *interactomen* te preserveren (zowel qua functionaliteit als qua samenstelling) om biotechnologische toepassingen mogelijk te maken.

In deel 1 werd vastgesteld dat $^{13}CH_4$ differentieel verdeeld wordt over de partners van het *interactoom* doorheen de tijd. We suggereerden dat de non-MOB partners die het sterkst verweven waren in het *interactoom* de eersten gingen zijn om $^{13}CH_4$-C in hun DNA in te bouwen, en bijgevolg werden deze partners beschouwd als "primaire consumenten" in tegenstelling tot partners die slechts later gelabeld werden doorheen het SIP experiment en daardoor in losser verband staan met de MOB en als "secundaire consumenten" van organische koolstof afkomstig van de MOB en primaire consumenten beschouwd kunnen worden. In deel 2a vonden we geen adaptatie-effect van een matig competitieve partner aan de MOB (of vice versa) met een gammaproteobacteriële MOB, maar een duidelijke (doch beperkte) aanpassing kon worden waargenomen met een alfaproteobacteriële MOB. Een derde partner slaagde er nagenoeg steeds in om de non-MOB partner te verdringen uit een reeds gevestigde MOB:non-MOB interactie, ongeacht de initiële compatibiliteit van deze partner met de MOB. In deel 2b toonden we een duidelijke partnerselectie aan waar sommige persistente partners "promiscue" waren en konden persisteren ongeacht het type MOB waar ze mee samen groeiden terwijl andere persisterende partners meer specifiek gekoppeld waren met specifieke types MOB. Aanvullend demonstreerden we dat alfa- en gamma-proteobacteriële MOB CH_4-C op een verschillende manier verdeelden naar het methanotrofe interactoom. Uiteindelijk toonden we aan dat er geen significante invloed van co-cultivatie met non-MOB kon gevonden worden voor de grootte van de methaanoxidatiesnelheid, hoewel co-cultivatie erin slaagde om de biologische variabiliteit in de MOR te stabiliseren in vergelijking met axenische MOB. In deel 3 konden we dankzij het geoptimaliseerde

cryopreservatieprotocol voor gemengde microbiële culturen op een adequate manier zowel de gemeenschapsstructuur als functionaliteit van methanotrofe (en andere) *interactomen* preserveren voor 3 maanden bij -80°C.

Deze bevindingen kunnen van belang zijn voor microbieel resource management van methanotrofe *interactomen*, zeker als biotechnologische toepassingen van non-MOB partners het einddoel vormen. In het algemeen waren ongecoördineerde methodes voor de samenstelling van synthetische microbiële *interactomen* beter dan gecoördineerde omdat ze specifiek enkel non-MOB partners selecteren die kunnen samenleven met de MOB. Als specifieke biotechnologische toepassingen gevonden kunnen worden voor deze optimale methanotrofe *interactomen*, dan is het van belang om een reproduceerbare stockcultuur te kunnen bewaren voor langere tijdsduur. Het exploiteren en regelen van methanotrofe *interactomen* zou kunnen leiden tot een verbeterede en duurzame inperking en terugwinning van methaan, zowel onder de vorm van metabolische energie als methaan-koolstof in duurzame, biologisch geproduceerde eindproducten.

References

REFERENCES

Aben, R. C. H. V., M.; Helmsing, N.R.; van de Waal, D.B.; Lamers, L.P.M.; Kosten, S. (2016). Climate change effects on greenhouse gas balances of shallow lakes. KNVM Spring meeting 2016 - session: "The role of microorganisms in biogeochemical cycling and climate change", 22/03/2016, Papendal, Arnhem, The Netherlands, Van Zuiden Communications BV

Aberer, A. J., Pattengale, N. D. and Stamatakis, A. (2010). "Parallelized phylogenetic post-analysis on multi-core architectures." Journal of Computational Science **1**(2): 107-114.

Abrudan, M. I., Smakman, F., Grimbergen, A. J., Westhoff, S., Miller, E. L., van Wezel, G. P. and Rozen, D. E. (2015). "Socially mediated induction and suppression of antibiosis during bacterial coexistence." Proceedings of the National Academy of Sciences of the United States of America **112**(35): 11054-11059.

Acha, V., Alba, J. and Thalasso, F. (2002). "The absolute requirement for carbon dioxide for aerobic methane oxidation by a methanotrophic-heterotrophic soil community of bacteria." Biotechnology Letters **24**(9): 675-679.

Ackermann, M. (2015). "A functional perspective on phenotypic heterogeneity in microorganisms." Nature Reviews Microbiology **13**(8): 497-508.

Agasild, H., Zingel, P., Tuvikene, L., Tuvikene, A., Timm, H., Feldmann, T., Salujoe, J., Toming, K., Jones, R. I. and Noges, T. (2014). "Biogenic methane contributes to the food web of a large, shallow lake." Freshwater Biology **59**(2): 272-285.

Agler, M. T., Wrenn, B. A., Zinder, S. H. and Angenent, L. T. (2011). "Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform." Trends in Biotechnology **29**(2): 70-78.

Amaral, J. A., Archambault, C., Richards, S. R. and Knowles, R. (1995). "Denitrification associated with groups I and II methanotrophs in a gradient enrichment system." FEMS Microbiology Ecology **18**(4): 289-298.

Anderson, M. J., Ellingsen, K. E. and McArdle, B. H. (2006). "Multivariate dispersion as a measure of beta diversity." Ecology Letters **9**(6): 683-693.

Angenent, L. T., Karim, K., Al-Dahhan, M. H. and Domiguez-Espinosa, R. (2004). "Production of bioenergy and biochemicals from industrial and agricultural wastewater." Trends in Biotechnology **22**(9): 477-485.

Anthony, C. (1982). Biochemistry of methylotrophs. London, Academic Press.

Anthony, C. (1991). Assimilation of carbon by methylotrophs. In: Biology of Methylotrophs. I. Goldberg and J. S. Rokem.

Stoneham, MA, Butterworth-Heinemann**:** 79-109.

Antony, C. P., Kumaresan, D., Ferrando, L., Boden, R., Moussard, H., Scavino, A. F., Shouche, Y. S. and Murrell, J. C. (2010). "Active methylotrophs in the sediments of Lonar Lake, a saline and alkaline ecosystem formed by meteor impact." The ISME Journal **4**(11): 1470-1480.

Anvar, S. Y., Frank, J., Pol, A., Schmitz, A., Kraaijeveld, K., den Dunnen, J. T. and den Camp, H. J. M. O. (2014). "The genomic landscape of the verrucomicrobial methanotroph *Methylacidiphilum fumariolicum* SolV." BMC Genomics **15**(1). doi: 10.1186/1471-2164-15-914.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formsma, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G. D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A. and Zagnitko, O. (2008). "The RAST server: Rapid annotations using subsystems technology." BMC Genomics **9**(1): 75. doi: 10.1186/1471-2164-9-75.

Baani, M. and Liesack, W. (2008). "Two isozymes of particulate methane monooxygenase with different methane oxidation kinetics are found in *Methylocystis* sp. strain SCZ." Proceedings of the National Academy of Sciences of the United States of America **105**(29): 10203-10208.

Bai, Y., Müller, D. B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., Dombrowski, N., Münch, P. C., Spaepen, S. and Remus-Emsermann, M. (2015). "Functional overlap of the Arabidopsis leaf and root microbiota." Nature **528**(7582): 364-369.

Balasubramanian, R., Levinson, B. T. and Rosenzweig, A. C. (2010). "Secretion of flavins by three species of methanotrophic bacteria." Applied and Environmental Microbiology **76**(21): 7356-7358.

Balasubramanian, R. and Rosenzweig, A. C. (2008). "Copper methanobactin: a molecule whose time has come." Current Opinion in Chemical Biology **12**(2): 245-249.

Banda, N., Krol, M., van Weele, M., van Noije, T. and Rockmann, T. (2013). "Analysis of global methane changes after the 1991 Pinatubo volcanic eruption." Atmospheric Chemistry and Physics **13**(4): 2267-2281.

Bao, Z., Okubo, T., Kubota, K., Kasahara, Y., Tsurumaru, H., Anda, M., Ikeda, S. and Minamisawa, K. (2014). "Metaproteomic identification of diazotrophic methanotrophs and their localization in root tissues of field-grown rice plants." Applied and Environmental Microbiology **80**(16): 5043-5052.

Beal, E. J., House, C. H. and Orphan, V. J. (2009). "Manganese- and Iron-dependent marine methane oxidation." Science **325**(5937): 184-187.

Beck, D. A. C., Kalyuzhnaya, M. G., Malfatti, S., Tringe, S. G., del Rio, T. G., Ivanova, N., Lidstrom, M. E. and Chistoserdova, L. (2013). "A metagenomic insight into freshwater methane-utilizing communities and evidence for cooperation between the Methylococcaceae and the

Methylophilaceae." PeerJ **1**: e23. doi: 10.7717/peerj.23.

Beck, D. A. C., McTaggart, T. L., Setboonsarng, U., Vorobev, A., Kalyuzhnaya, M. G., Ivanova, N., Goodwin, L., Woyke, T., Lidstrom, M. E. and Chistoserdova, L. (2014). "The Expanded Diversity of *Methylophilaceae* from Lake Washington through Cultivation and Genomic Sequencing of Novel Ecotypes." PLOS ONE **9**(7) e102458.

Bell, T., Newman, J. A., Silverman, B. W., Turner, S. L. and Lilley, A. K. (2005). "The contribution of species richness and composition to bacterial services." Nature **436**(7054): 1157-1160.

Belova, S. E., Baani, M., Suzina, N. E., Bodelier, P. L. E., Liesack, W. and Dedysh, S. N. (2011). "Acetate utilization as a survival strategy of peat-inhabiting Methylocystis spp." Environmental Microbiology Reports **3**(1): 36-46.

Benner, J., De Smet, D., Ho, A., Kerckhof, F.-M., Vanhaecke, L., Heylen, K. and Boon, N. (2015). "Exploring methane-oxidizing communities for the co-metabolic degradation of organic micropollutants." Applied Microbiology and Biotechnology **99**(8): 3609-3618.

Berry, D., Mader, E., Lee, T. K., Woebken, D., Wang, Y., Zhu, D., Palatinszky, M., Schintimeister, A., Schmid, M. C., Hanson, B. T., Shterzer, N., Mizrahi, I., Rauch, I., Decker, T., Bocklitz, T., Popp, J., Gibson, C. M., Fowler, P. W., Huang, W. E. and Wagner, M. (2015). "Tracking heavy water (D2O) incorporation for identifying and sorting active microbial cells." Proceedings of the National Academy of Sciences of the United States of America **112**(2): E194-E203.

Blanksby, S. J. and Ellison, G. B. (2003). "Bond dissociation energies of organic molecules." Accounts of Chemical Research **36**(4): 255-263.

Boden, R., Thomas, E., Savani, P., Kelly, D. P. and Wood, A. P. (2008). "Novel methylotrophic bacteria isolated from the River Thames (London, UK)." Environmental Microbiology **10**(12): 3225-3236.

Bodrossy, L., Stralis-Pavese, N., Konrad-Koszler, M., Weilharter, A., Reichenauer, T. G., Schofer, D. and Sessitsch, A. (2006). "mRNA-based parallel detection of active methanotroph populations by use of a diagnostic microarray." Applied and Environmental Microbiology **72**(2): 1672-1676.

Bohorquez, L., Delgado-Serrano, L., López, G., Osorio-Forero, C., Klepac-Ceraj, V., Kolter, R., Junca, H., Baena, S. and Zambrano, M. (2012). "In-depth characterization via complementing culture-independent approaches of the microbial community in an acidic hot spring of the Colombian Andes." Microbial Ecology **63**(1): 103-115.

Boon, N., De Windt, W., Verstraete, W. and Top, E. M. (2002). "Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants." FEMS Microbiology Ecology **39**(2): 101-112.

Boon, N., Goris, J., De Vos, P., Verstraete, W. and Top, E. M. (2000). "Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosteroni* strain, I2gfp." Applied and Environmental Microbiology **66**(7): 2906-2913.

Bosch, G., Wang, T., Latypova, E., Kalyuzhnaya, M. G., Hackett, M. and Chistoserdova, L. (2009). "Insights into the physiology of *Methylotenera mobilis* as revealed by metagenome-based shotgun proteomic analysis." Microbiology **155**(4): 1103-1110.

Bothe, H., Jensen, K. M., Mergel, A., Larsen, J., Jorgensen, C., Bothe, H. and Jorgensen, L. (2002). "Heterotrophic bacteria growing in association with *Methylococcus capsulatus* (Bath) in a single cell protein production process." Applied Microbiology and Biotechnology **59**(1): 33-39.

Bowman, J. (2006). The methanotrophs—the families Methylococcaceae and Methylocystaceae. In: The Prokaryotes, Springer**:** 266-289.

Bowman, J. P. (2014). The family Methylococcaceae. In: The Prokaryotes, Springer**:** 411-440.

Brenner, K., You, L. C. and Arnold, F. H. (2008). "Engineering microbial consortia: a new frontier in synthetic biology." Trends in Biotechnology **26**(9): 483-489.

Bussmann, I., Rahalkar, M. and Schink, B. (2006). "Cultivation of methanotrophic bacteria in opposing gradients of methane and oxygen." FEMS Microbiology Ecology **56**(3): 331-344.

But, S. Y., Khmelenina, V. N., Reshetnikov, A. S., Mustakhimov, I. I., Kalyuzhnaya, M. G. and Trotsenko, Y. A. (2015). "Sucrose metabolism in halotolerant methanotroph *Methylomicrobium alcaliphilum* 20Z." Archives of Microbiology **197**(3): 471-480.

But, S. Y., Rozova, O. N., Khmelenina, V. N., Reshetnikov, A. S. and Trotsenko, Y. A. (2012). "Properties of Recombinant ATP-Dependent Fructokinase from the Halotolerant Methanotroph *Methylomicrobium alcaliphilum* 20Z." Biochemistry-Moscow **77**(4): 372-377.

Cabezas, A., de Araujo, J. C., Callejas, C., Gales, A., Hamelin, J., Marone, A., Sousa, D. Z., Trably, E. and Etchebehere, C. (2015). "How to use molecular biology tools for the study of the anaerobic digestion process?" Reviews in Environmental Science and Bio-Technology **14**(4): 555-593.

Camarinha-Silva, A., Jáuregui, R., Chaves-Moreno, D., Oxley, A., Schaumburg, F., Becker, K., Wos-Oxley, M. and Pieper, D. (2014). "Comparing the anterior nare bacterial community of two discrete human populations using Illumina amplicon sequencing." Environmental Microbiology **16**(9): 2939-2952.

Casper, J. K. (2010). Greenhouse gases: worldwide impacts. New York, Facts on File (Infobase Publishing).

Cayrou, C., Sambe, B., Armougom, F., Raoult, D. and Drancourt, M. (2013). "Molecular diversity of the Planctomycetes

in the human gut microbiota in France and Senegal." APMIS **121**(11): 1082-1090.

Cebron, A., Bodrossy, L., Chen, Y., Singer, A. C., Thompson, I. P., Prosser, J. I. and Murrell, J. C. (2007). "Identity of active methanotrophs in landfill cover soil as revealed by DNA-stable isotope probing." FEMS Microbiology Ecology **62**(1): 12-23.

Cebron, A., Bodrossy, L., Stralis-Pavese, N., Singer, A. C., Thompson, I. P., Prosser, J. I. and Murrell, J. C. (2007). "Nutrient amendments in soil DNA stable isotope probing experiments reduce the observed methanotroph diversity." Applied and Environmental Microbiology **73**(3): 798-807.

Chen, Y., Wu, L. Q., Boden, R., Hillebrand, A., Kumaresan, D., Moussard, H., Baciu, M., Lu, Y. H. and Murrell, J. C. (2009). "Life without light: microbial diversity and evidence of sulfur- and ammonium-based chemolithotrophy in Movile Cave." The ISME Journal **3**(9): 1093-1104.

Chiellini, C., Munz, G., Petroni, G., Lubello, C., Mori, G., Verni, F. and Vannini, C. (2013). "Characterization and comparison of bacterial communities selected in conventional activated sludge and membrane bioreactor pilot plants: A focus on Nitrospira and Planctomycetes bacterial phyla." Current Microbiology **67**(1): 77-90.

Chistoserdova, L. (2011). "Modularity of methylotrophy, revisited." Environmental Microbiology **13**(10): 2603-2622.

Chistoserdova, L., Kalyuzhnaya, M. G. and Lidstrom, M. E. (2009). "The expanding world of methylotrophic metabolism." Annual Review of Microbiology **63**: 477-499.

Chistoserdova, L., Lapidus, A., Han, C., Goodwin, L., Saunders, L., Brettin, T., Tapia, R., Gilna, P., Lucas, S. and Richardson, P. M. (2007). "Genome of *Methylobacillus flagellatus*, molecular basis for obligate methylotrophy, and polyphyletic origin of methylotrophy." Journal of Bacteriology **189**(11): 4020-4027.

Chu, F. and Lidstrom, M. E. (2016). "XoxF Acts as the Predominant Methanol Dehydrogenase in the Type I Methanotroph *Methylomicrobium buryatense*." Journal of Bacteriology **198**(8): 1317-1325.

Ciais, P., Sabine, C., Bala, G., Bopp, L., Brovkin, V., Canadell, J., Chhabra, A., DeFries, R., Galloway, J. and Heimann, M. (2014). Carbon and other biogeochemical cycles. In: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, Cambridge University Press**:** 465-570.

Cole, J. R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R. J., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Marsh, T., Garrity, G. M. and Tiedje, J. M. (2009). "The Ribosomal Database Project: improved alignments and new tools for rRNA analysis." Nucleic Acids Research **37**(Suppl 1): D141-D145.

Conrad, R. (2007). "Microbial ecology of methanogens and methanotrophs." Advances in Agronomy **96**: 1-63.

Conrad, R. (2009). "The global methane cycle: recent advances in understanding the microbial processes involved." Environmental Microbiology Reports **1**(5): 285-292.

Costa, C., Dijkema, C., Friedrich, M., Garcia-Encina, P., Fernandez-Polanco, F. and Stams, A. J. M. (2000). "Denitrification with methane as electron donor in oxygen-limited bioreactors." Applied Microbiology and Biotechnology **53**(6): 754-762.

Costa, E., Perez, J. and Kreft, J. U. (2006). "Why is metabolic labour divided in nitrification?" Trends in Microbiology **14**(5): 213-219.

Crombie, A. T. and Murrell, J. C. (2014). "Trace-gas metabolic versatility of the facultative methanotroph *Methylocella silvestris*." Nature **510**(7503): 148-151.

Culpepper, M. A. and Rosenzweig, A. C. (2012). "Architecture and active site of particulate methane monooxygenase." Critical Reviews in Biochemistry and Molecular Biology **47**(6): 483-492.

D'Onofrio, A., Crawford, J. M., Stewart, E. J., Witt, K., Gavrish, E., Epstein, S., Clardy, J. and Lewis, K. (2010). "Siderophores from Neighboring Organisms Promote the Growth of Uncultured Bacteria." Chemistry & Biology **17**(3): 254-264.

Daebeler, A., Bodelier, P. L. E., Yan, Z., Hefting, M. M., Jia, Z. J. and Laanbroek, H. J. (2014). "Interactions between Thaumarchaea, Nitrospira and methanotrophs modulate autotrophic nitrification in volcanic grassland soil." The ISME Journal **8**(12): 2397-2410.

Dalsøren, S. B., Myhre, C. L., Myhre, G., Gomez-Pelaez, A. J., Søvde, O. A., Isaksen, I. S., Weiss, R. F. and Harth, C. M. (2016). "Atmospheric methane evolution the last 40 years." Atmospheric Chemistry and Physics **16**(5): 3099-3126.

Dalton, H. (2005). "The Leeuwenhoek Lecture 2000 - The natural and unnatural history of methane-oxidizing bacteria." Philosophical Transactions of the Royal Society B-Biological Sciences **360**(1458): 1207-1222.

Danilova, O. V., Kulichevskaya, I. S., Rozova, O. N., Detkova, E. N., Bodelier, P. L. E., Trotsenko, Y. A. and Dedysh, S. N. (2013). "*Methylomonas paludis* sp. nov., the first acid-tolerant member of the genus *Methylomonas*, from an acidic wetland." International Journal of Systematic and Evolutionary Microbiology **63**: 2282-2289.

Danilova, O. V., Suzina, N. E., Van De Kamp, J., Svenning, M. M., Bodrossy, L. and Dedysh, S. N. (2016). "A new cell morphotype among methane oxidizers: a spiral-shaped obligately microaerophilic methanotroph from northern low-oxygen environments." The ISME Journal, 10.1038/ismej.2016.48.

Davidson, C. J. and Surette, M. G. (2008). "Individuality in Bacteria." Annual Review of Genetics **42**: 253-268.

De Clippeleir, H., Courtens, E., Mosquera, M., Vlaeminck, S. E., Smets, B. F., Boon, N. and Verstraete, W. (2012). "Efficient total

nitrogen removal in an ammonia gas biofilter through high-rate OLAND." Environmental Science & Technology **46**(16): 8826-8833.

de Jong, I. G., Veening, J. W. and Kuipers, O. P. (2012). "Single cell analysis of gene expression patterns during carbon starvation in *Bacillus subtilis* reveals large phenotypic variation." Environmental Microbiology **14**(12): 3110-3121.

De Roy, K., Marzorati, M., Van den Abbeele, P., Van de Wiele, T. and Boon, N. (2014). "Synthetic microbial ecosystems: an exciting tool to understand and apply microbial communities." Environmental Microbiology **16**(6): 1472-1481.

De Schryver, P., Sinha, A. K., Kunwar, P. S., Baruah, K., Verstraete, W., Boon, N., De Boeck, G. and Bossier, P. (2010). "Poly-beta-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, Dicentrarchus labrax." Applied Microbiology and Biotechnology **86**(5): 1535-1541.

De Weirdt, R., Possemiers, S., Vermeulen, G., Moerdijk-Poortvliet, T. C. W., Boschker, H. T. S., Verstraete, W. and Van de Wiele, T. (2010). "Human faecal microbiota display variable patterns of glycerol metabolism." FEMS Microbiology Ecology **74**(3): 601-611.

Dedysh, S. N. (2009). "Exploring methanotroph diversity in acidic northern wetlands: Molecular and cultivation-based studies." Microbiology **78**(6): 655-669.

Dedysh, S. N., Knief, C. and Dunfield, P. F. (2005). "*Methylocella* species are facultatively methanotrophic." Journal of Bacteriology **187**(13): 4665-4670.

Dedysh, S. N., Kulichevskaya, I. S., Serkebaeva, Y. M., Mityaeva, M. A., Sorokin, V. V., Suzina, N. E., Rijpstra, W. I. and Damste, J. S. (2012). "*Bryocella elongata* gen. nov., sp. nov., a member of subdivision 1 of the Acidobacteria isolated from a methanotrophic enrichment culture, and emended description of *Edaphobacter aggregans* Koch et al. 2008." International Journal of Systematic and Evolutionary Microbiology **62**(3): 654-664.

Dejonghe, W., Boon, N., Seghers, D., Top, E. M. and Verstraete, W. (2001). "Bioaugmentation of soils by increasing microbial richness: missing links." Environmental Microbiology **3**(10): 649-657.

Demirel, B., Scherer, P., Yenigun, O. and Onay, T. T. (2010). "Production of methane and hydrogen from biomass through conventional and high-rate anaerobic digestion processes." Critical Reviews in Environmental Science and Technology **40**(2): 116-146.

Deutzmann, J. S., Hoppert, M. and Schink, B. (2014). "Characterization and phylogeny of a novel methanotroph, *Methyloglobulus morosus* gen. nov., spec. nov." Systematic and Applied Microbiology **37**(3): 165-169.

Dinh, T. N., Wille, M., De Schryver, P., Defoirdt, T., Bossier, P. and Sorgeloos, P. (2010). "The effect of poly beta-hydroxybutyrate on larviculture of the giant freshwater prawn *Macrobrachium rosenbergii*." Aquaculture **302**(1-2): 76-81.

DiSpirito, A. A., Semrau, J. D., Murrell, J. C., Gallagher, W. H., Dennison, C. and Vuilleumier, S. (2016). "Methanobactin and the link between copper and bacterial methane oxidation." Microbiology and Molecular Biology Reviews **80**(2): 387-409.

Doronina, N., Kaparullina, E. and Trotsenko, Y. (2014). The Family *Methylophilaceae*. In: The Prokaryotes: Alphaproteobacteria and Betaproteobacteria, Springer**:** 869-880.

Duan, C. H., Luo, M. F. and Xing, X. H. (2011). "High-rate conversion of methane to methanol by *Methylosinus trichosporium* OB3b." Bioresource Technology **102**(15): 7349-7353.

Duan, Y. F., Al-Soud, W. A., Brejnrod, A., Sorensen, S. J., Elsgaard, L., Petersen, S. O. and Boon, N. (2014). "Methanotrophs, methanogens, and microbial community structure in livestock slurry surface crusts." Journal of Applied Microbiology **117**(4): 1066-1078.

Dubilier, N., Bergin, C. and Lott, C. (2008). "Symbiotic diversity in marine animals: the art of harnessing chemosynthesis." Nature Reviews Microbiology **6**(10): 725-740.

Dufrêne, M. and Legendre, P. (1997). "Species assemblages and indicator species: the need for a flexible asymmetrical approach." Ecological monographs **67**(3): 345-366.

Dumont, M. G. (2014). Primers: functional marker genes for methylotrophs and methanotrophs. In: Hydrocarbon and Lipid Microbiology Protocols. T. J. McGenity, K. N. Timmis and B. Nogales. Berlin Heidelberg, Springer-Verlag, 10.1007/8623_2014_23**:** 1-21.

Dumont, M. G. and Murrell, J. C. (2005). "Stable isotope probing - linking microbial identity to function." Nature Reviews Microbiology **3**(6): 499-504.

Dumont, M. G., Pommerenke, B. and Casper, P. (2013). "Using stable isotope probing to obtain a targeted metatranscriptome of aerobic methanotrophs in lake sediment." Environmental Microbiology Reports **5**(5): 757-764.

Dumont, M. G., Pommerenke, B., Casper, P. and Conrad, R. (2011). "DNA-, rRNA- and mRNA-based stable isotope probing of aerobic methanotrophs in lake sediment." Environmental Microbiology **13**(5): 1153-1167.

Dunfield, P. F., Belova, S. E., Vorob'ev, A. V., Cornish, S. L. and Dedysh, S. N. (2010). "*Methylocapsa aurea* sp. nov., a facultative methanotroph possessing a particulate methane monooxygenase, and emended description of the genus *Methylocapsa*." International Journal of Systematic and Evolutionary Microbiology **60**: 2659-2664.

Dunfield, P. F., Liesack, W., Henckel, T., Knowles, R. and Conrad, R. (1999). "High-affinity methane oxidation by a soil enrichment culture containing a type II methanotroph." Applied and Environmental Microbiology **65**(3): 1009-1014.

Dunfield, P. F., Yimga, M. T., Dedysh, S. N., Berger, U., Liesack, W. and Heyer, J. (2002). "Isolation of a *Methylocystis* strain

containing a novel pmoA-like gene." FEMS Microbiology Ecology **41**(1): 17-26.

Dunfield, P. F., Yuryev, A., Senin, P., Smirnova, A. V., Stott, M. B., Hou, S. B., Ly, B., Saw, J. H., Zhou, Z. M., Ren, Y., Wang, J. M., Mountain, B. W., Crowe, M. A., Weatherby, T. M., Bodelier, P. L. E., Liesack, W., Feng, L., Wang, L. and Alam, M. (2007). "Methane oxidation by an extremely acidophilic bacterium of the phylum Verrucomicrobia." Nature **450**(7171): 879-882.

Egli, K., Bosshard, F., Werlen, C., Lais, P., Siegrist, H., Zehnder, A. J. B. and van der Meer, J. R. (2003). "Microbial composition and structure of a rotating biological contactor biofilm treating ammonium-rich wastewater without organic carbon." Microbial Ecology **45**(4): 419-432.

Eisentraeger, A., Klag, P., Vansbotter, B., Heymann, E. and Dott, W. (2001). "Denitrification of groundwater with methane as sole hydrogen donor." Water Research **35**(9): 2261-2267.

El Fantroussi, S., Verschuere, L., Verstraete, W. and Top, E. M. (1999). "Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles." Applied and Environmental Microbiology **65**(3): 982-988.

Eller, G., Kruger, M. and Frenzel, P. (2005). "Comparing field and microcosm experiments: a case study on methano- and methylo-trophic bacteria in paddy soil." FEMS Microbiology Ecology **51**(2): 279-291.

Emerson, D. and Wilson, W. (2009). "Giving microbial diversity a home." Nature Reviews Microbiology **7**(11): 758.

Epstein, S. S. (2013). "The phenomenon of microbial uncultivability." Current opinion in microbiology **16**(5): 636-642.

Esser, D. S., Leveau, J. H. and Meyer, K. M. (2015). "Modeling microbial growth and dynamics." Applied Microbiology and Biotechnology **99**(21): 8831-8846.

Estrada, J. M., Lebrero, R., Quijano, G., Perez, R., Figueroa-Gonzalez, I., Garcia-Encina, P. A. and Munoz, R. (2014). "Methane abatement in a gas-recycling biotrickling filter: Evaluating innovative operational strategies to overcome mass transfer limitations." Chemical Engineering Journal **253**: 385-393.

Ettwig, K. F., van Alen, T., van de Pas-Schoonen, K. T., Jetten, M. S. M. and Strous, M. (2009). "Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum." Applied and Environmental Microbiology **75**(11): 3656-3662.

Eyice, Ö. and Schäfer, H. (2016). "Culture-dependent and culture-independent methods reveal diverse methylotrophic communities in terrestrial environments." Archives of Microbiology **198**(1): 17-26.

Faith, J. J., Ahern, P. P., Ridaura, V. K., Cheng, J. and Gordon, J. I. (2014). "Identifying gut microbe–host phenotype relationships using combinatorial communities in gnotobiotic mice." Science

translational medicine **6**(220): 220ra211-220ra211.

Fei, Q., Guarnieri, M. T., Tao, L., Laurens, L. M., Dowe, N. and Pienkos, P. T. (2014). "Bioconversion of natural gas to liquid fuel: Opportunities and challenges." Biotechnology Advances **32**(3): 596-614.

Fiegna, F., Moreno-Letelier, A., Bell, T. and Barraclough, T. G. (2015). "Evolution of species interactions determines microbial community productivity in new environments." The ISME Journal **9**(5): 1235-1245.

Foster, K. R. and Bell, T. (2012). "Competition, not cooperation, dominates interactions among culturable microbial species." Current Biology **22**(19): 1845-1850.

Fuller, B. J. (2004). "Cryoprotectants: The essential antifreezes to protect life in the frozen state." Cryoletters **25**(6): 375-388.

Ganendra, G., De Muynck, W., Ho, A., Hoefman, S., De Vos, P., Boeckx, P. and Boon, N. (2014). "Atmospheric methane removal by methane-oxidizing bacteria immobilized on porous building materials." Applied Microbiology and Biotechnology **98**(8): 3791-3800.

Ganendra, G., Mercado-Garcia, D., Hernandez-Sanabria, E., Peiren, N., De Campeneere, S., Ho, A. and Boon, N. (2015). "Biofiltration of methane from ruminants gas effluent using Autoclaved Aerated Concrete as the carrier material." Chemical Engineering Journal **277**: 318-323.

Garrett, R. A. and Klenk, H.-P. (2008). Archaea: evolution, physiology, and molecular biology. Malden, MA, John Wiley & Sons.

Geymonat, E., Ferrando, L. and Tarlera, S. E. (2011). "*Methylogaea oryzae* gen. nov., sp nov., a mesophilic methanotroph isolated from a rice paddy field." International Journal of Systematic and Evolutionary Microbiology **61**: 2568-2572.

Graef, C., Hestnes, A. G., Svenning, M. M. and Frenzel, P. (2011). "The active methanotrophic community in a wetland from the High Arctic." Environmental Microbiology Reports **3**(4): 466-472.

Greenberg, A. E., Eaton, A. D., Clesceri, L. S. and Franson, M. A. H. (1992). Standard Methods for the Examination of Water and Wastewater, American Public Health Association.

Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G. and Bailey, M. J. (2000). "Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition." Applied and Environmental Microbiology **66**(12): 5488-5491.

Grime, J. P. (1977). "Evidence for the existence of three primary strategies in plants and its relevance to ecological and evolutionary theory." American naturalist: 1169-1194.

Großkopf, T. and Soyer, O. S. (2014). "Synthetic microbial communities." Current opinion in microbiology **18**: 72-77.

Hakemian, A. S. and Rosenzweig, A. C. (2007). "The biochemistry of methane oxidation." Annual Review of Biochemistry **76**: 223-241.

Hamer, G. (2010). "Methanotrophy: From the environment to industry and back." Chemical Engineering Journal **160**(2): 391-397.

Hamilton, M. J., Weingarden, A. R., Sadowsky, M. J. and Khoruts, A. (2012). "Standardized frozen preparation for transplantation of fecal microbiota for recurrent *Clostridium difficile* infection." American Journal of Gastroenterology **107**(5): 761-767.

Han, B., Chen, Y., Abell, G., Jiang, H., Bodrossy, L., Zhao, J. G., Murrell, J. C. and Xing, X. H. (2009). "Diversity and activity of methanotrophs in alkaline soil from a Chinese coal mine." FEMS Microbiology Ecology **70**(2): 196-207.

Han, B., Su, T., Wu, H., Gou, Z. X., Xing, X. H., Jiang, H., Chen, Y., Li, X. and Murrell, J. C. (2009). "Paraffin oil as a "methane vector" for rapid and high cell density cultivation of *Methylosinus trichosporium* OB3b." Applied Microbiology and Biotechnology **83**(4): 669-677.

Hanke, A., Hamann, E., Sharma, R., Geelhoed, J. S., Hargesheimer, T., Kraft, B., Meyer, V., Lenk, S., Osmers, H., Wu, R., Makinwa, K., Hettich, R. L., Banfield, J. F., Tegetmeyer, H. E. and Strous, M. (2014). "Recoding of the stop codon UGA to glycine by a BD1-5/SN-2 bacterium and niche partitioning between Alpha- and Gammaproteobacteria in a tidal sediment microbial community naturally selected in a laboratory chemostat." Frontiers in Microbiology **5**: 231. doi: 10.3389/fmicb.2014.00231.

Hanson, R. S. (1980). "Ecology and diversity of methylotrophic organisms." Advances in applied microbiology **26**(3): 3-39.

Hanson, R. S. and Hanson, T. E. (1996). "Methanotrophic bacteria." Microbiological Reviews **60**(2): 439-471.

Haroon, M. F., Hu, S. H., Shi, Y., Imelfort, M., Keller, J., Hugenholtz, P., Yuan, Z. G. and Tyson, G. W. (2013). "Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage." Nature **500**(7464): 567-570.

Harwood, J. and Pirt, S. (1972). "Quantitative aspects of growth of the methane oxidizing bacterium *Methylococcus capsulatus* on methane in shake flask and continuous chemostat culture." Journal of Applied Bacteriology **35**(4): 597-607.

He, R., Wooller, M. J., Pohlman, J. W., Catranis, C., Quensen, J., Tiedje, J. M. and Leigh, M. B. (2012). "Identification of functionally active aerobic methanotrophs in sediments from an arctic lake using stable isotope probing." Environmental Microbiology **14**(6): 1403-1419.

He, R., Wooller, M. J., Pohlman, J. W., Quensen, J., Tiedje, J. M. and Leigh, M. B. (2012). "Diversity of active aerobic methanotrophs along depth profiles of arctic and subarctic lake water column and

sediments." The ISME Journal **6**(10): 1937-1948.

He, R., Wooller, M. J., Pohlman, J. W., Quensen, J., Tiedje, J. M. and Leigh, M. B. (2012). "Shifts in identity and activity of methanotrophs in arctic lake sediments in response to temperature changes." Applied and Environmental Microbiology **78**(13): 4715-4723.

Helm, J., Wendlandt, K. D., Rogge, G. and Kappelmeyer, U. (2006). "Characterizing a stable methane-utilizing mixed culture used in the synthesis of a high-quality biopolymer in an open system." Journal of Applied Microbiology **101**(2): 387-395.

Henckel, T., Roslev, P. and Conrad, R. (2000). "Effects of O_2 and CH_4 on presence and activity of the indigenous methanotrophic community in rice field soil." Environmental Microbiology **2**(6): 666-679.

Hermans, I., Spier, E. S., Neuenschwander, U., Turra, N. and Baiker, A. (2009). "Selective oxidation catalysis: opportunities and challenges." Topics in Catalysis **52**(9): 1162-1174.

Hernandez, M. E., Beck, D. A., Lidstrom, M. E. and Chistoserdova, L. (2015). "Oxygen availability is a major factor in determining the composition of microbial communities involved in methane oxidation." PeerJ **3**: e801. doi: 10.7717/peerj.801.

Hesselsoe, M., Boysen, S., Iversen, N., Jorgensen, L., Murrell, J. C., McDonald, I., Radajewski, S., Thestrup, H. and Roslev, P. (2005). "Degradation of organic pollutants by methane grown microbial consortia." Biodegradation **16**(5): 435-448.

Heylen, K., Ettwig, K., Hu, Z. Y., Jetten, M. and Kartal, B. (2012). "Rapid and simple cryopreservation of anaerobic ammonium-oxidizing bacteria." Applied and Environmental Microbiology **78**(8): 3010-3013.

Heylen, K., Hoefman, S., Vekeman, B., Peiren, J. and De Vos, P. (2012). "Safeguarding bacterial resources promotes biotechnological innovation." Applied Microbiology and Biotechnology **94**(3): 565-574.

Hilger, H. A., Cranford, D. F. and Barlaz, M. A. (2000). "Methane oxidation and microbial exopolymer production in landfill cover soil." Soil Biology & Biochemistry **32**(4): 457-467.

Hinrichs, K.-U. and Boetius, A. (2002). The anaerobic oxidation of methane: new insights in microbial ecology and biogeochemistry. In: Ocean Margin Systems, Springer**:** 457-477.

Hirayama, H., Abe, M., Miyazaki, M., Nunoura, T., Furushima, Y., Yamamoto, H. and Takai, K. (2014). "*Methylomarinovum caldicuralii* gen. nov., sp nov., a moderately thermophilic methanotroph isolated from a shallow submarine hydrothermal system, and proposal of the family *Methylothermaceae* fam. nov." International Journal of Systematic and Evolutionary Microbiology **64**: 989-999.

Hirayama, H., Fuse, H., Abe, M., Miyazaki, M., Nakamura, T., Nunoura, T., Furushima,

Y., Yamamoto, H. and Takai, K. (2013). "*Methylomarinum vadi* gen. nov., sp nov., a methanotroph isolated from two distinct marine environments." International Journal of Systematic and Evolutionary Microbiology **63**: 1073-1082.

Ho, A. and Bodelier, P. L. E. (2015). "Diazotrophic methanotrophs in peatlands: the missing link?" Plant and Soil **389**(1-2): 419-423.

Ho, A., de Roy, K., Thas, O., De Neve, J., Hoefman, S., Vandamme, P., Heylen, K. and Boon, N. (2014). "The more, the merrier: heterotroph richness stimulates methanotrophic activity." The ISME Journal **8**(9): 1945-1948.

Ho, A., Kerckhof, F.-M., Lüke, C., Reim, A., Krause, S., Boon, N. and Bodelier, P. L. E. (2013). "Conceptualizing functional traits and ecological characteristics of methane-oxidizing bacteria as life strategies." Environmental Microbiology Reports **5**(3): 335-345.

Ho, A., Lüke, C. and Frenzel, P. (2011). "Recovery of methanotrophs from disturbance: population dynamics, evenness and functioning." The ISME Journal **5**(4): 750-758.

Ho, A., van den Brink, E., Reim, A., Krause, S. M. and Bodelier, P. L. (2016). "Recurrence and frequency of disturbance have cumulative effect on methanotrophic activity, abundance, and community structure." Frontiers in Microbiology **6**: 1493. doi: 10.3389/fmicb.2015.01493.

Ho, A., Vlaeminck, S. E., Ettwig, K. F., Schneider, B., Frenzel, P. and Boon, N. (2013). "Revisiting Methanotrophic Communities in Sewage Treatment Plants." Applied and Environmental Microbiology **79**(8): 2841-2846.

Hoefman, S., Pommerening-Roser, A., Samyn, E., De Vos, P. and Heylen, K. (2013). "Efficient cryopreservation protocol enables accessibility of a broad range of ammonia-oxidizing bacteria for the scientific community." Research in Microbiology **164**(4): 288-292.

Hoefman, S., van der Ha, D., Boon, N., Vandamme, P., De Vos, P. and Heylen, K. (2014). "Customized media based on miniaturized screening improve growth rate and cell yield of methane-oxidizing bacteria of the genus Methylomonas." Antonie Van Leeuwenhoek **105**(2): 353-366.

Hoefman, S., van der Ha, D., Boon, N., Vandamme, P., De Vos, P. and Heylen, K. (2014). "Niche differentiation in nitrogen metabolism among methanotrophs within an operational taxonomic unit." BMC Microbiology **14**(1): 83. doi: 10.1186/1471-2180-14-83.

Hoefman, S., van der Ha, D., De Vos, P., Boon, N. and Heylen, K. (2012). "Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria." Microbial Biotechnology **5**(3): 368-378.

Hoefman, S., van der Ha, D., Iguchi, H., Yurimoto, H., Sakai, Y., Boon, N., Vandamme, P., Heylen, K. and De Vos, P. (2014). "*Methyloparacoccus murrellii* gen. nova, sp nov., a methanotroph isolated from pond water." International Journal of

Systematic and Evolutionary Microbiology **64**: 2100-2107.

Hoefman, S., Van Hoorde, K., Boon, N., Vandamme, P., De Vos, P. and Heylen, K. (2012). "Survival or revival: long-term preservation induces a reversible viable but non-culturable state in methane-oxidizing bacteria." PLOS ONE **7**(4). doi: 10.1371/journal.pone.0034196.

Hollister, E. B., Forrest, A. K., Wilkinson, H. H., Ebbole, D. J., Malfatti, S. A., Tringe, S. G., Holtzapple, M. T. and Gentry, T. J. (2010). "Structure and dynamics of the microbial communities underlying the carboxylate platform for biofuel production." Applied Microbiology and Biotechnology **88**(1): 389-399.

Holmes, A. J., Owens, N. J. P. and Murrell, J. C. (1995). "Detection of novel marine methanotrophs using phylogenetic and functional gene probes after methane enrichment." Microbiology-Uk **141**: 1947-1955.

Hou, S. B., Makarova, K. S., Saw, J. H. W., Senin, P., Ly, B. V., Zhou, Z. M., Ren, Y., Wang, J. M., Galperin, M. Y., Omelchenko, M. V., Wolf, Y. I., Yutin, N., Koonin, E. V., Stott, M. B., Mountain, B. W., Crowe, M. A., Smirnova, A. V., Dunfield, P. F., Feng, L., Wang, L. and Alam, M. (2008). "Complete genome sequence of the extremely acidophilic methanotroph isolate V4, *Methylacidiphilum infernorum*, a representative of the bacterial phylum Verrucomicrobia." Biology Direct **3**(1), 1-25. doi: 10.1186/1745-6150-3-26.

Hrsak, D. and Begonja, A. (1998). "Growth characteristics and metabolic activities of the methanotrophic-heterotrophic groundwater community." Journal of Applied Microbiology **85**(3): 448-456.

Hrsak, D. and Begonja, A. (2000). "Possible interactions within a methanotrophic-heterotrophic groundwater community able to transform linear alkylbenzenesulfonates." Applied and Environmental Microbiology **66**(10): 4433-4439.

Huang, W. E., Stoecker, K., Griffiths, R., Newbold, L., Daims, H., Whiteley, A. S. and Wagner, M. (2007). "Raman-FISH: combining stable-isotope Raman spectroscopy and fluorescence in situ hybridization for the single cell analysis of identity and function." Environmental Microbiology **9**(8): 1878-1889.

Hubálek, Z. (2003). "Protectants used in the cryopreservation of microorganisms." Cryobiology **46**(3): 205-229.

Huber-Humer, M., Gebert, J. and Hilger, H. (2008). "Biotic systems to mitigate landfill methane emissions." Waste Management & Research **26**(1): 33-46.

Hung, W.-L., Wade, W. G., Boden, R., Kelly, D. P. and Wood, A. P. (2011). "Facultative methylotrophs from the human oral cavity and methylotrophy in strains of Gordonia, Leifsonia, and Microbacterium." Archives of Microbiology **193**(6): 407-417.

Huq, M. N., Ralph, B. J. and Rickard, P. A. D. (1978). "The extracellular polysaccharide of a methylotrophic culture." Australian Journal of Biological Sciences **31**(3): 311-316.

Hutchens, E., Radajewski, S., Dumont, M. G., McDonald, I. R. and Murrell, J. C. (2004). "Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing." Environmental Microbiology **6**(2): 111-120.

Iguchi, H., Yurimoto, H. and Sakai, Y. (2011). "*Methylovulum miyakonense* gen. nov., sp. nov., a type I methanotroph isolated from forest soil." International Journal of Systematic and Evolutionary Microbiology **61**: 810-815.

Iguchi, H., Yurimoto, H. and Sakai, Y. (2011). "Stimulation of methanotrophic growth in cocultures by cobalamin excreted by rhizobia." Applied and Environmental Microbiology **77**(24): 8509-8515.

Iguchi, H., Yurimoto, H. and Sakai, Y. (2015). "Interactions of methylotrophs with plants and other heterotrophic bacteria." Microorganisms **3**(2): 137-151.

Im, J., Lee, S. W., Yoon, S., DiSpirito, A. A. and Semrau, J. D. (2011). "Characterization of a novel facultative *Methylocystis* species capable of growth on methane, acetate and ethanol." Environmental Microbiology Reports **3**(2): 174-181.

Im, J. and Semrau, J. D. (2011). "Pollutant degradation by a *Methylocystis* strain SB2 grown on ethanol: bioremediation via facultative methanotrophy." FEMS Microbiology Letters **318**(2): 137-142.

Jagmann, N. and Philipp, B. (2014). "Design of synthetic microbial communities for biotechnological production processes." Journal of Biotechnology **184**: 209-218.

Jardine, C. N., Boardman, B., Osman, A., Vowles, J. and Palmer, J. (2004). methane UK. Oxford, Environmental Change Institute, University of Oxford.

Jensen, S., Neufeld, J. D., Birkeland, N. K., Hovland, M. and Murrell, J. C. (2008). "Methane assimilation and trophic interactions with marine *Methylomicrobium* in deep-water coral reef sediment off the coast of Norway." FEMS Microbiology Letters **66**(2): 320-330.

Jeong, S.-Y., Cho, K.-S. and Kim, T. G. (2014). "Density-dependent enhancement of methane oxidation activity and growth of *Methylocystis* sp. by a non-methanotrophic bacterium *Sphingopyxis* sp." Biotechnology Reports **4**: 128-133.

Jiang, H., Chen, Y., Jiang, P. X., Zhang, C., Smith, T. J., Murrell, J. C. and Xing, X. H. (2010). "Methanotrophs: Multifunctional bacteria with promising applications in environmental bioengineering." Biochemical Engineering Journal **49**(3): 277-288.

Jimenez-Infante, F., Ngugi, D. K., Vinu, M., Alam, I., Kamau, A. A., Blom, J., Bajic, V. B. and Stingl, U. (2016). "Comprehensive genomic analyses of the OM43 clade, including a novel species from the Red Sea, indicate ecotype differentiation among marine methylotrophs." Applied and Environmental Microbiology **82**(4): 1215-1226.

Johnson, Z. I., Zinser, E. R., Coe, A., McNulty, N. P., Woodward, E. M. S. and Chisholm, S. W. (2006). "Niche partitioning among *Prochlorococcus* ecotypes along ocean-scale environmental gradients." Science **311**(5768): 1737-1740.

Jousset, A., Schulz, W., Scheu, S. and Eisenhauer, N. (2011). "Intraspecific genotypic richness and relatedness predict the invasibility of microbial communities." The ISME Journal **5**(7): 1108-1114.

Joye, S. B. (2012). "A piece of the methane puzzle." Nature **491**(7425): 538-539.

Kaeberlein, T., Lewis, K. and Epstein, S. S. (2002). "Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment." Science **296**(5570): 1127-1129.

Kalyuzhnaya, M. G., Bowerman, S., Lara, J. C., Lidstrom, M. E. and Chistoserdova, L. (2006). "*Methylotenera mobilis* gen. nov., sp nov., an obligately methylamine-utilizing bacterium within the family *Methylophilaceae*." International Journal of Systematic and Evolutionary Microbiology **56**: 2819-2823.

Kalyuzhnaya, M. G., Hristova, K. R., Lidstrom, M. E. and Chistoserdova, L. (2008). "Characterization of a novel methanol dehydrogenase in representatives of Burkholderiales: Implications for environmental detection of methylotrophy and evidence for convergent evolution." Journal of Bacteriology **190**(11): 3817-3823.

Kalyuzhnaya, M. G., Lapidus, A., Ivanova, N., Copeland, A. C., McHardy, A. C., Szeto, E., Salamov, A., Grigoriev, I. V., Suciu, D. and Levine, S. R. (2008). "High-resolution metagenomics targets specific functional types in complex microbial communities." Nature biotechnology **26**(9): 1029-1034.

Kalyuzhnaya, M. G., Yang, S., Rozova, O. N., Smalley, N. E., Clubb, J., Lamb, A., Gowda, G. A. N., Raftery, D., Fu, Y., Bringel, F., Vuilleumier, S., Beck, D. A. C., Trotsenko, Y. A., Khmelenina, V. N. and Lidstrom, M. E. (2013). "Highly efficient methane biocatalysis revealed in a methanotrophic bacterium." Nature Communications **4**: 2785. doi: 10.1038/ncomms3785.

Kandel, R. and Viollier, M. (2005). "Planetary radiation budgets." Space Science Reviews **120**(1-2): 1-26.

Karp, P. D., Paley, S. M., Krummenacker, M., Latendresse, M., Dale, J. M., Lee, T. J., Kaipa, P., Gilham, F., Spaulding, A., Popescu, L., Altman, T., Paulsen, I., Keseler, I. M. and Caspi, R. (2010). "Pathway Tools version 13.0: integrated software for pathway/genome informatics and systems biology." Briefings in Bioinformatics **11**(1): 40-79.

Keltjens, J. T., Pol, A., Reimann, J. and Op den Camp, H. J. M. (2014). "PQQ-dependent methanol dehydrogenases: rare-earth elements make a difference." Applied Microbiology and Biotechnology **98**(14): 6163-6183.

Kerckhof, F. M., Courtens, E. N. P., Geirnaert, A., Hoefman, S., Ho, A., Vilchez-Vargas, R., Pieper, D. H., Jauregui, R., Vlaeminck, S. E., Van de Wiele, T., Vandamme, P., Heylen, K. and Boon, N. (2014). "Optimized cryopreservation of mixed microbial communities for conserved functionality and diversity." PLOS ONE **9**(6). doi: 10.1371/journal.pone.0099517.

Khadem, A. F., Pol, A., Wieczorek, A., Mohammadi, S. S., Francoijs, K. J.,

Stunnenberg, H. G., Jetten, M. S. M. and Op den Camp, H. J. M. (2011). "Autotrophic Methanotrophy in Verrucomicrobia: *Methylacidiphilum fumariolicum* SolV Uses the Calvin-Benson-Bassham Cycle for Carbon Dioxide Fixation." Journal of Bacteriology **193**(17): 4438-4446.

Khandelwal, R. A., Olivier, B. G., Roling, W. F. M., Teusink, B. and Bruggeman, F. J. (2013). "Community flux balance analysis for microbial consortia at balanced growth." PLOS ONE **8**(5). doi: 10.1371/journal.pone.0064567.

Khmelenina, V., Rozova, O., But, S. Y., Mustakhimov, I., Reshetnikov, A., Beschastnyi, A. and Trotsenko, Y. A. (2015). "Biosynthesis of secondary metabolites in methanotrophs: Biochemical and genetic aspects (Review)." Applied Biochemistry and Microbiology **51**(2): 150-158.

Kim, H. J., Graham, D. W., DiSpirito, A. A., Alterman, M. A., Galeva, N., Larive, C. K., Asunskis, D. and Sherwood, P. M. (2004). "Methanobactin, a copper-acquisition compound from methane-oxidizing bacteria." Science **305**(5690): 1612-1615.

Kip, N., van Winden, J. F., Pan, Y., Bodrossy, L., Reichart, G. J., Smolders, A. J. P., Jetten, M. S. M., Damste, J. S. S. and Op den Camp, H. J. M. (2010). "Global prevalence of methane oxidation by symbiotic bacteria in peat-moss ecosystems." Nature Geoscience **3**(9): 617-621.

Kirschke, S., Bousquet, P., Ciais, P., Saunois, M., Canadell, J. G., Dlugokencky, E. J., Bergamaschi, P., Bergmann, D., Blake, D. R., Bruhwiler, L., Cameron-Smith, P., Castaldi, S., Chevallier, F., Feng, L., Fraser, A., Heimann, M., Hodson, E. L., Houweling, S., Josse, B., Fraser, P. J., Krummel, P. B., Lamarque, J. F., Langenfelds, R. L., Le Quere, C., Naik, V., O'Doherty, S., Palmer, P. I., Pison, I., Plummer, D., Poulter, B., Prinn, R. G., Rigby, M., Ringeval, B., Santini, M., Schmidt, M., Shindell, D. T., Simpson, I. J., Spahni, R., Steele, L. P., Strode, S. A., Sudo, K., Szopa, S., van der Werf, G. R., Voulgarakis, A., van Weele, M., Weiss, R. F., Williams, J. E. and Zeng, G. (2013). "Three decades of global methane sources and sinks." Nature Geoscience **6**(10): 813-823.

Kits, K. D., Klotz, M. G. and Stein, L. Y. (2015). "Methane oxidation coupled to nitrate reduction under hypoxia by the Gammaproteobacterium *Methylomonas denitrificans*, sp nov type strain FJG1." Environmental Microbiology **17**(9): 3219-3232.

Kleerebezem, R. and van Loosdrecht, M. C. M. (2007). "Mixed culture biotechnology for bioenergy production." Current Opinion in Biotechnology **18**(3): 207-212.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glöckner, F. O. (2013). "Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies." Nucleic Acids Research **41**(1): e1. doi: 10.1093/nar/gks808.

Knief, C. (2015). "Diversity and habitat preferences of cultivated and uncultivated aerobic methanotrophic bacteria evaluated based on *pmoA* as molecular marker." Frontiers in Microbiology **6**: 1346. doi: 10.3389/fmicb.2015.01346.

Knittel, K. and Boetius, A. (2009). "Anaerobic oxidation of methane: progress with an unknown process." Annual Review of Microbiology **63**: 311-334.

Koch, H., Lucker, S., Albertsen, M., Kitzinger, K., Herbold, C., Spieck, E., Nielsen, P. H., Wagner, M. and Daims, H. (2015). "Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus *Nitrospira*." Proceedings of the National Academy of Sciences of the United States of America **112**(36): 11371-11376.

Koch, I. H., Gich, F., Dunfield, P. F. and Overmann, J. (2008). "*Edaphobacter modestus* gen. nov., sp. nov., and *Edaphobacter aggregans* sp. nov., acidobacteria isolated from alpine and forest soils." International Journal of Systematic and Evolutionary Microbiology **58**(5): 1114-1122.

Kolb, S. (2009). "Aerobic methanol-oxidizing Bacteria in soil." FEMS Microbiology Letters **300**(1): 1-10.

Kolb, S. (2009). "The quest for atmospheric methane oxidizers in forest soils." Environmental Microbiology Reports **1**(5): 336-346.

Kolb, S., Knief, C., Stubner, S. and Conrad, R. (2003). "Quantitative detection of methanotrophs in soil by novel *pmoA*-targeted real-time PCR assays." Applied and Environmental Microbiology **69**(5): 2423-2429.

Konietschke, F. (2009). "Simultane Konfidenzintervalle fuer nichtparametrische relative Kontrasteffekte." University of Goettingen.

Konietschke, F. and Hothorn, L. A. (2012). "Rank-based multiple test procedures and simultaneous confidence intervals." Electronic Journal of Statistics **6**: 738-759.

Konietschke, F., Placzek, M., Schaarschmidt, F. and Hothorn, L. A. (2015). "nparcomp: An R Software Package for Nonparametric Multiple Comparisons and Simultaneous Confidence Intervals." Journal of Statistical Software **64**(9). doi: 10.18637/jss.v064.i09.

Kraft, B., Tegetmeyer, H. E., Sharma, R., Klotz, M. G., Ferdelman, T. G., Hettich, R. L., Geelhoed, J. S. and Strous, M. (2014). "The environmental controls that govern the end product of bacterial nitrate respiration." Science **345**(6197): 676-679.

Krause, S., Lüke, C. and Frenzel, P. (2012). "Methane source strength and energy flow shape methanotrophic communities in oxygen-methane counter-gradients." Environmental Microbiology Reports **4**(2): 203-208.

Kravchenko, I. K., Kizilova, A. K., Bykova, S. A., Men'ko, E. V. and Gal'chenko, V. F. (2010). "Molecular analysis of high-affinity methane-oxidizing enrichment cultures isolated from a forest biocenosis and agrocenoses." Microbiology **79**(1): 106-114.

Kuai, L. P. and Verstraete, W. (1998). "Ammonium removal by the oxygen-limited autotrophic nitrification-denitrification system." Applied and Environmental Microbiology **64**(11): 4500-4506.

Lamb, S. and Garver, J. (1980). "Interspecific interactions in a methane - utilizing mixed culture." Biotechnology and Bioengineering **22**(10): 2119-2135.

Lamb, S. C. and Garver, J. C. (1980). "Batch-culture and continuous-culture studies of a methane-utilizing mixed culture." Biotechnology and Bioengineering **22**(10): 2097-2118.

Lapidus, A., Clum, A., LaButti, K., Kaluzhnaya, M. G., Lim, S., Beck, D. A., del Rio, T. G., Nolan, M., Mavromatis, K. and Huntemann, M. (2011). "Genomes of three methylotrophs from a single niche reveal the genetic and metabolic divergence of the *Methylophilaceae*." Journal of Bacteriology **193**(15): 3757-3764.

Larmola, T., Leppanen, S. M., Tuittila, E. S., Aarva, M., Merila, P., Fritze, H. and Tiirola, M. (2014). "Methanotrophy induces nitrogen fixation during peatland development." Proceedings of the National Academy of Sciences of the United States of America **111**(2): 734-739.

Larsen, P., Hamada, Y. and Gilbert, J. (2012). "Modeling microbial communities: Current, developing, and future technologies for predicting microbial community interaction." Journal of Biotechnology **160**(1-2): 17-24.

Lattermann, C. and Büchs, J. (2015). "Microscale and miniscale fermentation and screening." Current Opinion in Biotechnology **35**: 1-6.

Laurin, V., Labbe, V., Juteau, P., Parent, S. and Villemur, R. (2006). "Long-term storage conditions for carriers with denitrifying biomass of the fluidized, methanol-fed denitrification reactor of the Montreal Biodome, and the impact on denitrifying activity and bacterial population." Water Research **40**(9): 1836-1840.

Lee, J. M., Gianchandani, E. P. and Papin, J. A. (2006). "Flux balance analysis in the era of metabolomics." Briefings in Bioinformatics **7**(2): 140-150.

Letunic, I. and Bork, P. (2011). "Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy." Nucleic Acids Research **39**, W475-W478. doi: doi: 10.1093/Nar/Gkr201.

Lidstrom, M. (2006). Aerobic Methylotrophic Prokaryotes. In: The Prokaryotes. M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer and E. Stackebrandt, Springer New York. **2:** 618-634.

Lidstrom, M. E. and Konopka, M. C. (2010). "The role of physiological heterogeneity in microbial population behavior." Nature Chemical Biology **6**(10): 705-712.

Liebner, S., Zeyer, J., Wagner, D., Schubert, C., Pfeiffer, E. M. and Knoblauch, C. (2011). "Methane oxidation associated with submerged brown mosses reduces methane emissions from Siberian polygonal tundra." Journal of Ecology **99**(4): 914-922.

Lin, J. L., Radajewski, S., Eshinimaev, B. T., Trotsenko, Y. A., McDonald, I. R. and Murrell, J. C. (2004). "Molecular diversity of methanotrophs in Transbaikal soda lake sediments and identification of potentially

active populations by stable isotope probing." Environmental Microbiology **6**(10): 1049-1060.

Linton, J. D. and Buckee, J. C. (1977). "Interactions in a methane-utilizing mixed bacterial culture in a chemostat." Journal of General Microbiology **101**(Aug): 219-225.

Liu, J. J., Sun, F. Q., Wang, L., Ju, X., Wu, W. X. and Chen, Y. X. (2014). "Molecular characterization of a microbial consortium involved in methane oxidation coupled to denitrification under micro-aerobic conditions." Microbial Biotechnology **7**(1): 64-76.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Buchner, A., Lai, T., Steppi, S., Jobb, G. and Förster, W. (2004). "ARB: a software environment for sequence data." Nucleic Acids Research **32**(4): 1363-1371.

Luesken, F. A., Sanchez, J., van Alen, T. A., Sanabria, J., Op den Camp, H. J. M., Jetten, M. S. M. and Kartal, B. (2011). "Simultaneous nitrite-dependent anaerobic methane and ammonium oxidation processes." Applied and Environmental Microbiology **77**(19): 6802-6807.

Lüke, C. and Frenzel, P. (2011). "Potential of *pmoA* Amplicon Pyrosequencing for Methanotroph Diversity Studies." Applied and Environmental Microbiology **77**(17): 6305-6309.

Madhaiyan, M., Poonguzhali, S., Lee, J.-S., Lee, K. C. and Sundaram, S. (2010). "*Flavobacterium glycines* sp. nov., a facultative methylotroph isolated from the rhizosphere of soybean." International Journal of Systematic and Evolutionary Microbiology **60**(9): 2187-2192.

Malashenko, Y. R., Pirog, T. P., Romanovskaya, V. A., Sokolov, I. G. and Grinberg, T. A. (2001). "Search for methanotrophic producers of exopolysaccharides." Applied Biochemistry and Microbiology **37**(6): 599-602.

Marshall, C. W., LaBelle, E. V. and May, H. D. (2013). "Production of fuels and chemicals from waste by microbiomes." Current Opinion in Biotechnology **24**(3): 391-397.

Martineau, C., Whyte, L. G. and Greer, C. W. (2010). "Stable isotope probing analysis of the diversity and activity of methanotrophic bacteria in soils from the Canadian high Arctic." Applied and Environmental Microbiology **76**(17): 5773-5784.

Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D. and Verstraete, W. (2008). "How to get more out of molecular fingerprints: practical tools for microbial ecology." Environmental Microbiology **10**(6): 1571-1581.

Matsen, J. B., Yang, S., Stein, L. Y., Beck, D. and Kalyuzhnaya, M. G. (2013). "Global molecular analyses of methane metabolism in methanotrophic alphaproteobacterium, Methylosinus trichosporium OB3b. Part I: transcriptomic study." Frontiers in Microbiology **4**: 40. doi: 10.3389/fmicb.2013.00040.

McGlynn, S. E., Chadwick, G. L., Kempes, C. P. and Orphan, V. J. (2015). "Single cell activity reveals direct electron transfer in methanotrophic consortia." Nature **526**(7574): 531-535.

Medvedkova, K. A., Khmelenina, V. N. and Trotsenko, Y. A. (2007). "Sucrose as a factor of thermal adaptation of the thermophilic methanotroph *Methylocaldum szegediense* O-12." Microbiology **76**(4): 500-502.

Mee, M. T. and Wang, H. H. (2012). "Engineering ecosystems and synthetic ecologies." Molecular BioSystems **8**(10): 2470-2483.

Megraw, S. R. and Knowles, R. (1989). "Methane-dependent nitrate production by a microbial consortium enriched from a cultivated humisol." FEMS Microbiology Ecology **62**(6): 359-365.

Miller, A. W., Befort, C., Kerr, E. O. and Dunham, M. J. (2013). "Design and use of multiplexed chemostat arrays." Journal of Visualized Experiments, doi:10.3791/50262(72): e50262.

Milucka, J., Kirf, M., Lu, L., Krupke, A., Lam, P., Littmann, S., Kuypers, M. M. M. and Schubert, C. J. (2015). "Methane oxidation coupled to oxygenic photosynthesis in anoxic waters." The ISME Journal **9**(9): 1991-2002.

Minamisawa, K., Imaizumi-Anraku, H., Bao, Z., Shinoda, R., Okubo, T. and Ikeda, S. (2016). "Are symbiotic methanotrophs key microbes for N acquisition in paddy rice root?" Microbes and Environments **31**(1): 4-10.

Minshull, J., Ness, J., Purcell, T. J. and Leonard, E. (2014). "Compositions and methods for biological production of isoprene." Calysta Energy, Inc., Patent number:PCT/US2014/021258

Modin, O., Fukushi, K., Nakajima, F. and Yamamoto, K. (2010). "Aerobic methane oxidation coupled to denitrification: kinetics and effect of oxygen supply." Journal of Environmental Engineering **136**(2): 211-219.

Modin, O., Fukushi, K., Nakajima, F. and Yamamoto, K. (2010). "Nitrate removal and biofilm characteristics in methanotrophic membrane biofilm reactors with various gas supply regimes." Water Research **44**(1): 85-96.

Modin, O., Fukushi, K. and Yamamoto, K. (2007). "Denitrification with methane as external carbon source." Water Research **41**(12): 2726-2738.

Mohanty, S. R., Bodelier, P. L. E., Floris, V. and Conrad, R. (2006). "Differential effects of nitrogenous fertilizers on methane-consuming microbes in rice field and forest soils." Applied and Environmental Microbiology **72**(2): 1346-1354.

Moosvi, S. A., McDonald, I. R., Pearce, D. A., Kelly, D. P. and Wood, A. P. (2005). "Molecular detection and isolation from Antarctica of methylotrophic bacteria able to grow with methylated sulfur compounds." Systematic and Applied Microbiology **28**(6): 541-554.

Morris, J. J., Lenski, R. E. and Zinser, E. R. (2012). "The black queen hypothesis: Evolution of dependencies through adaptive gene loss." Mbio **3**(2): e00036-12. doi: doi:10.1128/mBio.00036-12.

Morris, S. A., Radajewski, S., Willison, T. W. and Murrell, J. C. (2002). "Identification of the functionally active methanotroph population in a peat soil microcosm by stable-isotope probing." Applied and Environmental Microbiology **68**(3): 1446-1453.

Moussard, H., Stralis-Pavese, N., Bodrossy, L., Neufeld, J. D. and Murrell, J. C. (2009). "Identification of active methylotrophic bacteria inhabiting surface sediment of a marine estuary." Environmental Microbiology Reports **1**(5): 424-433.

Muhamadali, H., Chisanga, M., Subaihi, A. and Goodacre, R. (2015). "Combining Raman and FT-IR spectroscopy with quantitative isotopic labeling for differentiation of *E. coli* cells at community and single cell levels." Analytical Chemistry **87**(8): 4578-4586.

Murase, J. and Frenzel, P. (2007). "A methane-driven microbial food web in a wetland rice soil." Environmental Microbiology **9**(12): 3025-3034.

Murase, J. and Frenzel, P. (2008). "Selective grazing of methanotrophs by protozoa in a rice field soil." FEMS Microbiology Ecology **65**(3): 408-414.

Muyzer, G., Dewaal, E. C. and Uitterlinden, A. G. (1993). "Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA." Applied and Environmental Microbiology **59**(3): 695-700.

Myhre, G., Shindell, D., Bréon, F., Collins, W., Fuglestvedt, J., Huang, J., Koch, D., Lamarque, J., Lee, D., Mendoza, B., Nakajima, T., Robock, A., Stephens, G., Takemura, T. and Zhang, H. (2013). 2013: Anthropogenic and natural radiative forcing. In: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge, Cambridge University Press**:** 659-740.

Namsaraev, B. and Zavarzin, T. (1972). "Trophic relationships in a methane-oxidizing culture." Microbiology **41**: 999-1006.

Nazaries, L., Murrell, J. C., Millard, P., Baggs, L. and Singh, B. K. (2013). "Methane, microbes and models: Fundamental understanding of the soil methane cycle for future predictions." Environmental Microbiology **15**(9): 2395-2417.

Neef, L., van Weele, M. and van Velthoven, P. (2010). "Optimal estimation of the present-day global methane budget." Global Biogeochemical Cycles **24**(4). doi: 10.1029/2009GB003661.

Nercessian, O., Bienvenu, N., Moreira, D., Prieur, D. and Jeanthon, C. (2005). "Diversity of functional genes of methanogens, methanotrophs and sulfate reducers in deep-sea hydrothermal environments." Environmental Microbiology **7**(1): 118-132.

directly coupled isotopic and phylogenetic analysis." Science **293**(5529): 484-487.

Neufeld, J. D., Vohra, J., Dumont, M. G., Lueders, T., Manefield, M., Friedrich, M. W. and Murrell, J. C. (2007). "DNA stable-isotope probing." Nature Protocols **2**(4): 860-866.

Osaka, T., Ebie, Y., Tsuneda, S. and Inamori, Y. (2008). "Identification of the bacterial community involved in methane-dependent denitrification in activated sludge using DNA stable-isotope probing." FEMS Microbiology Ecology **64**(3): 494-506.

Neufeld, J. D., Wagner, M. and Murrell, J. C. (2007). "Who eats what, where and when? Isotope-labelling experiments are coming of age." The ISME Journal **1**(2): 103-110.

Oshkin, I. Y., Beck, D. A. C., Lamb, A. E., Tchesnokova, V., Benuska, G., McTaggart, T. L., Kalyuzhnaya, M. G., Dedysh, S. N., Lidstrom, M. E. and Chistoserdova, L. (2015). "Methane-fed microbial microcosms show differential community dynamics and pinpoint taxa involved in communal response." The ISME Journal **9**(5): 1119-1129.

Nichols, D., Lewis, K., Orjala, J., Mo, S., Ortenberg, R., O'Connor, P., Zhao, C., Vouros, P., Kaeberlein, T. and Epstein, S. S. (2008). "Short peptide induces an "uncultivable" microorganism to grow *in vitro*." Applied and Environmental Microbiology **74**(15): 4889-4897.

Ott, M., Zola, J., Aluru, S. and Stamatakis, A. (2010). "Large-scale maximum likelihood-based phylogenetic analysis on the IBM BlueGene/L." 2007 Acm/Ieee Sc07 Conference: 466-476.

Nisbet, E. G., Dlugokencky, E. J. and Bousquet, P. (2014). "Methane on the rise-again." Science **343**(6170): 493-495.

Noll, M., Frenzel, P. and Conrad, R. (2008). "Selective stimulation of type I methanotrophs in a rice paddy soil by urea fertilization revealed by RNA-based stable isotope probing." FEMS Microbiology Ecology **65**(1): 125-132.

Overbeek, R., Olson, R., Pusch, G. D., Olsen, G. J., Davis, J. J., Disz, T., Edwards, R. A., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A. R., Xia, F. F. and Stevens, R. (2014). "The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST)." Nucleic Acids Research **42**(D1), D206-D214. doi: 10.1093/nar/gkt1226.

Nyerges, G., Han, S. K. and Stein, L. Y. (2010). "Effects of ammonium and nitrite on growth and competitive fitness of cultivated methanotrophic bacteria." Applied and Environmental Microbiology **76**(16): 5648-5651.

Ovreas, L., Forney, L., Daae, F. L. and Torsvik, V. (1997). "Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA." Applied and Environmental Microbiology **63**(9): 3367-3373.

Orphan, V. J., House, C. H., Hinrichs, K. U., McKeegan, K. D. and DeLong, E. F. (2001). "Methane-consuming archaea revealed by

Bioresource Technology **102**(21): 9919-9926.

Pande, S., Merker, H., Bohl, K., Reichelt, M., Schuster, S., de Figueiredo, L. F., Kaleta, C. and Kost, C. (2014). "Fitness and stability of obligate cross-feeding interactions that emerge upon gene loss in bacteria." The ISME Journal **8**(5): 953-962.

Pieja, A. J., Sundstrom, E. R. and Criddle, C. S. (2012). "Cyclic, alternating methane and nitrogen limitation increases PHB production in a methanotrophic community." Bioresource Technology **107**: 385-392.

Pandhal, J. and Noirel, J. (2014). "Synthetic microbial ecosystems for biotechnology." Biotechnology Letters **36**(6): 1141-1151.

Pilloni, G., Granitsiotis, M. S., Engel, M. and Lueders, T. (2012). "Testing the limits of 454 pyrotag sequencing: reproducibility, quantitative assessment and comparison to T-RFLP fingerprinting of aquifer microbes." PloS One **7**(7) e40467.

Pangala, S. R., Hornibrook, E. R. C., Gowing, D. J. and Gauci, V. (2015). "The contribution of trees to ecosystem methane emissions in a temperate forested wetland." Global Change Biology **21**(7): 2642-2654.

Possemiers, S., Grootaert, C., Vermeiren, J., Gross, G., Marzorati, M., Verstraete, W. and Van de Wiele, T. (2009). "The intestinal environment in health and disease - Recent insights on the potential of intestinal bacteria to influence human health." Current Pharmaceutical Design **15**(18): 2051-2065.

Pangala, S. R., Moore, S., Hornibrook, E. R. C. and Gauci, V. (2013). "Trees are major conduits for methane egress from tropical forested wetlands." The New Phytologist **197**(2): 524-531.

Prado-Rubio, O. A., Jørgensen, J. B. and Jørgensen, S. B. (2010). "Systematic Model Analysis for Single Cell Protein (SCP) Production in a U-Loop Reactor." Computer Aided Chemical Engineering **28**: 319-324.

Pattengale, N. D., Aberer, A. J., Swenson, K. M., Stamatakis, A. and Moret, B. M. E. (2011). "Uncovering Hidden Phylogenetic Consensus in Large Data Sets." IEE-ACM Transactions on Computational Biology and Bioinformatics **8**(4): 902-911.

Prakash, O., Nimonkar, Y. and Shouche, Y. S. (2013). "Practice and prospects of microbial preservation." FEMS Microbiology Letters **339**(1): 1-9.

Petersen, J. M. and Dubilier, N. (2009). "Methanotrophic symbioses in marine invertebrates." Environmental Microbiology Reports **1**(5): 319-335.

Prather, M. J., Holmes, C. D. and Hsu, J. (2012). "Reactive greenhouse gas scenarios: Systematic exploration of uncertainties and the role of atmospheric chemistry." Geophysical Research Letters **39**(9). doi: 10.1029/2012GL051440.

Pfluger, A. R., Wu, W. M., Pieja, A. J., Wan, J., Rostkowski, K. H. and Criddle, C. S. (2011). "Selection of Type I and Type II methanotrophic proteobacteria in a fluidized bed reactor under non-sterile conditions."

Pratt, C., Walcroft, A. S., Tate, K. R., Ross, D. J., Roy, R., Reid, M. H. and Veiga, P. W. (2012). "Biofiltration of methane emissions from a dairy farm effluent pond." Agriculture, Ecosystems & Environment **152**: 33-39.

Puri, A. W., Owen, S., Chu, F., Chavkin, T., Beck, D. A. C., Kalyuzhnaya, M. G. and Lidstrom, M. E. (2015). "Genetic tools for the industrially promising methanotroph *Methylomicrobium buryatense*." Applied and Environmental Microbiology **81**(5): 1766-1772.

Putkinen, A., Larmola, T., Tuomivirta, T., Siljanen, H. M. P., Bodrossy, L., Tuittila, E. S. and Fritze, H. (2014). "Peatland succession induces a shift in the community composition of Sphagnum-associated active methanotrophs." FEMS Microbiology Ecology **88**(3): 596-611.

Pynaert, K., Smets, B. F., Wyffels, S., Beheydt, D., Siciliano, S. D. and Verstraete, W. (2003). "Characterization of an autotrophic nitrogen-removing biofilm from a highly loaded lab-scale rotating biological contactor." Applied and Environmental Microbiology **69**(6): 3626-3635.

Qiu, Q., Conrad, R. and Lu, Y. (2009). "Cross‐feeding of methane carbon among bacteria on rice roots revealed by DNA‐stable isotope probing." Environmental Microbiology Reports **1**(5): 355-361.

Qiu, Q. F., Noll, M., Abraham, W. R., Lu, Y. H. and Conrad, R. (2008). "Applying stable isotope probing of phospholipid fatty acids and rRNA in a Chinese rice field to study activity and composition of the methanotrophic bacterial communities in situ." The ISME Journal **2**(6): 602-614.

Quayle, J. R. (1972). The metabolism of one-carbon compunds by microorganisms. In: Advances in microbial physiology. A. H. Rose and D. W. Tempest. London, Academic press. **7:** 119-205.

R Development Core Team (2015) "R: A language and environment for statistical computing" version 3.2.2

Radajewski, S., Webster, G., Reay, D. S., Morris, S. A., Ineson, P., Nedwell, D. B., Prosser, J. I. and Murrell, J. C. (2002). "Identification of active methylotroph populations in an acidic forest soil by stable-isotope probing." Microbiology **148**: 2331-2342.

Raghoebarsing, A. A., Smolders, A. J. P., Schmid, M. C., Rijpstra, W. I. C., Wolters-Arts, M., Derksen, J., Jetten, M. S. M., Schouten, S., Damste, J. S. S., Lamers, L. P. M., Roelofs, J. G. M., Op den Camp, H. J. M. and Strous, M. (2005). "Methanotrophic symbionts provide carbon for photosynthesis in peat bogs." Nature **436**(7054): 1153-1156.

Rasigraf, O., Kool, D. M., Jetten, M. S. M., Damste, J. S. S. and Ettwig, K. F. (2014). "Autotrophic carbon dioxide fxation via the Calvin-Benson-Bassham cycle by the denitrifying methanotroph "*Candidatus* Methylomirabilis oxyfera"." Applied and Environmental Microbiology **80**(8): 2451-2460.

Read, S., Marzorati, M., Guimaraes, B. C. M. and Boon, N. (2011). "Microbial Resource Management revisited: Successful

parameters and new concepts." Applied Microbiology and Biotechnology **90**(3): 861-871.

Rechner, A. R., Smith, M. A., Kuhnle, G., Gibson, G. R., Debnam, E. S., Srai, S. K. S., Moore, K. P. and Rice-Evans, C. A. (2004). "Colonic metabolism of dietary polyphenols: Influence of structure on microbial fermentation products." Free Radical Biology and Medicine **36**(2): 212-225.

Redmond, M. C., Valentine, D. L. and Sessions, A. L. (2010). "Identification of novel methane-, ethane-, and propane-oxidizing bacteria at marine hydrocarbon seeps by stable-isotope probing." Applied and Environmental Microbiology **76**(19): 6412-6422.

Reinhard, F. and van der Meer, J. R. (2013). "Improved statistical analysis of low abundance phenomena in bimodal bacterial populations." PLOS ONE **8**(10). doi: 10.1371/journal.pone.0078288.

Rhee, G. Y. and Fuhs, G. W. (1978). "Wastewater denitrification with one-carbon compounds as energy-source." Journal Water Pollution Control Federation **50**(9): 2111-2119.

Roberts, D. W. (2015) "labdsv: Ordination and multivariate analysis for ecology" version 1.7-0 R package

Rotaru, A. E. and Thamdrup, B. (2016). "A new diet for methane oxidizers." Science **351**(6274): 658-659.

Rothrock, M. J., Vanotti, M. B., Szogi, A. A., Gonzalez, M. C. G. and Fujii, T. (2011). "Long-term preservation of anammox bacteria." Applied Microbiology and Biotechnology **92**(1): 147-157.

Ruff, S. E., Arnds, J., Knittel, K., Amann, R., Wegener, G., Ramette, A. and Boetius, A. (2013). "Microbial communities of deep-sea methane seeps at Hikurangi continental margin (New Zealand)." PLOS ONE **8**(9). doi: 10.1371/journal.pone.0072627.

Saidi-Mehrabad, A., He, Z. G., Tamas, I., Sharp, C. E., Brady, A. L., Rochman, F. F., Bodrossy, L., Abell, G. C. J., Penner, T., Dong, X. L., Sensen, C. W. and Dunfield, P. F. (2013). "Methanotrophic bacteria in oilsands tailings ponds of northern Alberta." The ISME Journal **7**(5): 908-921.

Saleem, M., Fetzer, I., Dormann, C. F., Harms, H. and Chatzinotas, A. (2012). "Predator richness increases the effect of prey diversity on prey yield." Nature Communications **3**(1305), 1-7. doi: 10.1038/ncomms2287.

Sanseverino, A. M., Bastviken, D., Sundh, I., Pickova, J. and Enrich-Prast, A. (2012). "Methane carbon supports aquatic food webs to the fish level." PLOS ONE **7**(8). doi: 10.1371/journal.pone.0042723.

Schaefer, H., Fletcher, S. E. M., Veidt, C., Lassey, K. R., Brailsford, G. W., Bromley, T. M., Dlugokencky, E. J., Michel, S. E., Miller, J. B., Levin, I., Lowe, D. C., Martin, R. J., Vaughn, B. H. and White, J. W. C. (2016). "A 21st-century shift from fossil-fuel to biogenic methane emissions indicated by (CH4)-C-13." Science **352**(6281): 80-84.

Scheller, S., Yu, H., Chadwick, G. L., McGlynn, S. E. and Orphan, V. J. (2016). "Artificial electron acceptors decouple archaeal methane oxidation from sulfate reduction." Science **351**(6274): 703-707.

Scheutz, C., Kjeldsen, P., Bogner, J. E., De Visscher, A., Gebert, J., Hilger, H. A., Huber-Humer, M. and Spokas, K. (2009). "Microbial methane oxidation processes and technologies for mitigation of landfill gas emissions." Waste Management & Research **27**(5): 409-455.

Schloss, P. D., Gevers, D. and Westcott, S. L. (2011). "Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies." PLOS ONE **6**(12), e27310. doi: 10.1371/journal.pone.0027310.

Schloss, P. D., Westcott, S. L., Ryabin, T., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J. and Weber, C. F. (2009). "Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities." Applied and Environmental Microbiology **75**(23): 7537-7541.

Schuur, E. A. G., Abbott, B. W., Bowden, W. B., Brovkin, V., Camill, P., Canadell, J. G., Chanton, J. P., Chapin, F. S., Christensen, T. R., Ciais, P., Crosby, B. T., Czimczik, C. I., Grosse, G., Harden, J., Hayes, D. J., Hugelius, G., Jastrow, J. D., Jones, J. B., Kleinen, T., Koven, C. D., Krinner, G., Kuhry, P., Lawrence, D. M., McGuire, A. D., Natali, S. M., O'Donnell, J. A., Ping, C. L., Riley, W. J., Rinke, A., Romanovsky, V. E., Sannel, A. B. K., Schadel, C., Schaefer, K., Sky, J., Subin, Z. M., Tarnocai, C., Turetsky, M. R., Waldrop, M. P., Anthony, K. M. W., Wickland, K. P., Wilson, C. J. and Zimov, S. A. (2013). "Expert assessment of vulnerability of permafrost carbon to climate change." Climatic Change **119**(2): 359-374.

Semrau, J. D., Chistoserdov, A., Lebron, J., Costello, A., Davagnino, J., Kenna, E., Holmes, A. J., Finch, R., Murrell, J. C. and Lidstrom, M. E. (1995). "Particulate methane monooxygenase genes in methanotrophs." Journal of Bacteriology **177**(11): 3071-3079.

Semrau, J. D., DiSpirito, A. A. and Vuilleumier, S. (2011). "Facultative methanotrophy: False leads, true results, and suggestions for future research." FEMS Microbiology Letters **323**(1): 1-12.

Semrau, J. D., DiSpirito, A. A. and Yoon, S. (2010). "Methanotrophs and copper." FEMS Microbiology Reviews **34**(4): 496-531.

Sharp, C. E., Martinez-Lorenzo, A., Brady, A. L., Grasby, S. E. and Dunfield, P. F. (2014). "Methanotrophic bacteria in warm geothermal spring sediments identified using stable-isotope probing." FEMS Microbiology Ecology **90**(1): 92-102.

Sharp, C. E., Stott, M. B. and Dunfield, P. F. (2012). "Detection of autotrophic verrucomicrobial methanotrophs in a geothermal environment using stable isotope probing." Frontiers in Microbiology **3**: 303. doi: 10.3389/fmicb.2012.00303.

Smith, D. and Ryan, M. J. (2008). "The impact of OECD best practice on the validation of cryopreservation techniques for microorganisms." Cryo Letters **29**(1): 63-72.

Smith, T. J. and Murrell, C. J. (2011). Mutagenesis of soluble methane monooxygenase. In: Methods in Methane Metabolism, Part B: Methanotrophy. A. Rosenzweig and S. W. Ragsdale. **495:** 135-147.

Soo, R. M., Woodcroft, B. J., Parks, D. H., Tyson, G. W. and Hugenholtz, P. (2015). "Back from the dead; the curious tale of the predatory cyanobacterium *Vampirovibrio chlorellavorus*." PeerJ **3**, e968. doi: 10.7717/peerj.968.

Sorokin, D. Y. and Kuenen, J. G. (2005). "Chemolithotrophic halo alkaliphiles from soda lakes." FEMS Microbiology Ecology **52**(3): 287-295.

Sorokin, D. Y., Trotsenko, Y. A., Doronina, N. V., Tourova, T. P., Galinski, E. A., Kolganova, T. V. and Muyzer, G. (2007). "*Methylohalomonas lacus* gen. nov., sp nov and *Methylonatrum kenyense* gen. nov., sp nov., methylotrophic gammaproteobacteria from hypersaline lakes." International Journal of Systematic and Evolutionary Microbiology **57**: 2762-2769.

Stainthorpe, A. C., Lees, V., Salmond, G. P. C., Dalton, H. and Murrell, J. C. (1990). "The methane monooxygenase gene-cluster of *Methylococcus capsulatus* Bath." Gene **91**(1): 27-34.

Stamatakis, A. (2006). "RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models." Bioinformatics **22**(21): 2688-2690.

Stamatakis, A. (2014). "RAxML version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies." Bioinformatics **30**(9): 1312-1313.

Stenuit, B. and Agathos, S. N. (2015). "Deciphering microbial community robustness through synthetic ecology and molecular systems synecology." Current Opinion in Biotechnology **33**: 305-317.

Sternberg, C., Christensen, B. B., Johansen, T., Nielsen, A. T., Andersen, J. B., Givskov, M. and Molin, S. (1999). "Distribution of bacterial growth activity in flow-chamber biofilms." Applied and Environmental Microbiology **65**(9): 4108-4117.

Stock, M., Hoefman, S., Kerckhof, F.-M., Boon, N., De Vos, P., De Baets, B., Heylen, K. and Waegeman, W. (2013). "Exploration and prediction of interactions between methanotrophs and heterotrophs." Research in Microbiology **164**(10): 1045-1054.

Stoddard, S. F., Smith, B. J., Hein, R., Roller, B. R. and Schmidt, T. M. (2015). "rrnDB: improved tools for interpreting rRNA gene abundance in bacteria and archaea and a new foundation for future development." Nucleic Acids Research **43**(D1), D593-D598. doi: 10.1093/nar/gku1201.

Strong, P. J., Xie, S. and Clarke, W. P. (2015). "Methane as a resource: Can the methanotrophs add value?" Environmental Science & Technology **49**(7): 4001-4018.

Strovas, T. J. and Lidstrom, M. E. (2009). "Population heterogeneity in *Methylobacterium extorquens* AM1." Microbiology **155**: 2040-2048.

Su, Y., Xia, F.-F., Tian, B.-H., Li, W. and He, R. (2014). "Microbial community and function of enrichment cultures with methane and toluene." Applied Microbiology and Biotechnology **98**(7): 3121-3131.

Sun, F. Y., Dong, W. Y., Shao, M. F., Lv, X. M., Li, J., Peng, L. Y. and Wang, H. J. (2013). "Aerobic methane oxidation coupled to denitrification in a membrane biofilm reactor: Treatment performance and the effect of oxygen ventilation." Bioresource Technology **145**: 2-9.

Sun, J., Steindler, L., Thrash, J. C., Halsey, K. H., Smith, D. P., Carter, A. E., Landry, Z. C. and Giovannoni, S. J. (2011). "One carbon metabolism in SAR11 pelagic marine bacteria." PLOS ONE **6**(8), 1-12. doi: 10.1371/journal.pone.0023973.

Swings, T., Wuyts, S., Oeyen, E., Fauvart, M., Verstraeten, N. and Michiels, J. (2015). Plasticity of evolvability drives evolution to high ethanol tolerance in Escherichia coli (oral presentation). FEMS 6th congress of European Microbiologists. Maastricht.

Takeuchi, M., Kamagata, Y., Oshima, K., Hanada, S., Tamaki, H., Marumo, K., Maeda, H., Nedachi, M., Hattori, M., Iwasaki, W. and Sakata, S. (2014). "*Methylocaldum marinum* sp nov., a thermotolerant, methane-oxidizing bacterium isolated from marine sediments, and emended description of the genus *Methylocaldum*." International Journal of Systematic and Evolutionary Microbiology **64**: 3240-3246.

Tappe, W., Tomaschewski, C., Rittershaus, S. and Groeneweg, J. (1996). "Cultivation of nitrifying bacteria in the retentostat, a simple fermenter with internal biomass retention." FEMS Microbiology Ecology **19**(1): 47-52.

Tavormina, P. L., Hatzenpichler, R., McGlynn, S., Chadwick, G., Dawson, K. S., Connon, S. A. and Orphan, V. J. (2015). "*Methyloprofundus sedimenti* gen. nov., sp nov., an obligate methanotroph from ocean sediment belonging to the 'deep sea-1' clade of marine methanotrophs." International Journal of Systematic and Evolutionary Microbiology **65**: 251-259.

Tavormina, P. L., Orphan, V. J., Kalyuzhnaya, M. G., Jetten, M. S. M. and Klotz, M. G. (2011). "A novel family of functional operons encoding methane/ammonia monooxygenase-related proteins in gammaproteobacterial methanotrophs." Environmental Microbiology Reports **3**(1): 91-100.

Tinberg, C. E. and Lippard, S. J. (2011). "Dioxygen activation in soluble methane monooxygenase." Accounts of Chemical Research **44**(4): 280-288.

Trotsenko, Y. A. and Khmelenina, V. N. (2002). "Biology of extremophilic and extremotolerant methanotrophs." Archives of Microbiology **177**(2): 123-131.

Trotsenko, Y. A., Medvedkova, K. A., Khmelenina, V. N. and Eshinimayev, B. T. (2009). "Thermophilic and thermotolerant aerobic methanotrophs." Microbiology **78**(4): 387-401.

Trotsenko, Y. A. and Murrell, J. C. (2008). Metabolic aspects of aerobic obligate methanotrophy. In: Advances in applied microbiology, Academic Press. **63:** 183-229.

Turner, A., Jacob, D., Benmergui, J., Wofsy, S., Maasakkers, J., Butz, A., Hasekamp, O., Biraud, S. and Dlugokencky, E. (2016). "A large increase in US methane emissions over the past decade inferred from satellite data and surface observations." Geophysical Research Letters **43**(5): 2218-2224.

Tyc, O., van den Berg, M., Gerards, S., van Veen, J. A., Raaijmakers, J. M., de Boer, W. and Garbeva, P. (2014). "Impact of interspecific interactions on antimicrobial activity among soil bacteria." Frontiers in Microbiology **5**: 567. doi: 10.3389/fmicb.2014.00567.

Ul Haque, M. F., Kalidass, B., Bandow, N., Turpin, E. A., DiSpirito, A. A. and Semrau, J. D. (2015). "Cerium regulates expression of alternative methanol dehydrogenases in *Methylosinus trichosporium* OB3b." Applied and Environmental Microbiology **81**(21): 7546-7552.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R. and Leunissen, J. A. (2007). "Primer3Plus, an enhanced web interface to Primer3." Nucleic Acids Research **35**(suppl 2), W71-W74. doi: 10.1093/nar/gkm306

Usman, M., Daud, W. M. A. W. and Abbas, H. F. (2015). "Dry reforming of methane: Influence of process parameters - A review." Renewable & Sustainable Energy Reviews **45**: 710-744.

Van den Abbeele, P., Roos, S., Eeckhaut, V., MacKenzie, D. A., Derde, M., Verstraete, W., Marzorati, M., Possemiers, S., Vanhoecke, B., Van Immerseel, F. and Van de Wiele, T. (2012). "Incorporating a mucosal environment in a dynamic gut model results in a more representative colonization by lactobacilli." Microbial Biotechnology **5**(1): 106-115.

Van den Abbeele, P., Verstraete, W., El Aidy, S., Geirnaert, A. and Van de Wiele, T. (2013). "Prebiotics, faecal transplants and microbial network units to stimulate biodiversity of the human gut microbiome." Microbial Biotechnology **6**(4): 335-340.

van der Ha, D. (2013). "Methanotrophic microbiomes as drivers for environmental biotechnology." Gent, Department of Biochemical and microbial technology, Ghent University. **Doctoral dissertation (monograph)**

van der Ha, D., Bundervoet, B., Verstraete, W. and Boon, N. (2011). "A sustainable, carbon neutral methane oxidation by a partnership of methane oxidizing communities and microalgae." Water Research **45**(9): 2845-2854.

van der Ha, D., Hoefman, S., Boeckx, P., Verstraete, W. and Boon, N. (2010). "Copper enhances the activity and salt resistance of mixed methane-oxidizing communities." Applied Microbiology and Biotechnology **87**(6): 2355-2363.

van der Ha, D., Nachtergaele, L., Kerckhof, F.-M., Rameiyanti, D., Bossier, P., Verstraete, W. and Boon, N. (2012). "Conversion of biogas to bioproducts by algae and methane oxidizing bacteria." Environmental Science & Technology **46**(24): 13425-13431.

van der Ha, D., Vanwonterghem, I., Hoefman, S., De Vos, P. and Boon, N. (2013). "Selection of associated heterotrophs

by methane-oxidizing bacteria at different copper concentrations." Antonie Van Leeuwenhoek **103**(3): 527-537.

van Duinen, G. A., Vermonden, K., Bodelier, P. L. E., Hendriks, A. J., Leuven, R. S. E. W., Middelburg, J. J., van der Velde, G. and Verberk, W. C. E. P. (2013). "Methane as a carbon source for the food web in raised bog pools." Freshwater Science **32**(4): 1260-1272.

Van Nevel, S., Koetzsch, S., Weilenmann, H.-U., Boon, N. and Hammes, F. (2013). "Routine bacterial analysis with automated flow cytometry." Journal of Microbiological Methods **94**(2): 73-76.

van Teeseling, M. C. F., Pol, A., Harhangi, H. R., van der Zwart, S., Jetten, M. S. M. and den Camp, H. J. M. O. (2014). "Expanding the verrucomicrobial methanotrophic world: Description of three novel species of *Methylacidimicrobium* gen. nov." Applied and Environmental Microbiology **80**(21): 6782-6791.

Vekeman, B. (2016). "Methanotrophic microbiomes from North Sea sediment." Ghent, Faculty of Sciences, Laboratory of Microbiology (LM-UGent), Ghent University. **Doctoral thesis (Monograph)**

Vekeman, B., Hoefman, S., De Vos, P., Spieck, E. and Heylen, K. (2013). "A generally applicable cryopreservation method for nitrite-oxidizing bacteria." Systematic and Applied Microbiology **36**(8): 579-584.

Verstraete, W. (2015). "The manufacturing microbe." Microbial Biotechnology **8**(1): 36-37.

Verstraete, W., Wittelbolle, L., Heylen, K., Vanparys, B., de Vos, P., van de Wiele, T. and Boon, N. (2007). "Microbial resource management: The road to go for environmental biotechnology." Engineering in Life Sciences **7**(2): 117-126.

Vilchez‐Vargas, R., Geffers, R., Suárez‐Diez, M., Conte, I., Waliczek, A., Kaser, V. S., Kralova, M., Junca, H. and Pieper, D. H. (2013). "Analysis of the microbial gene landscape and transcriptome for aromatic pollutants and alkane degradation using a novel internally calibrated microarray system." Environmental Microbiology **15**(4): 1016-1039.

Vlaeminck, S. E., De Clippeleir, H. and Verstraete, W. (2012). "Microbial resource management of one-stage partial nitritation/anammox." Microbial Biotechnology **5**(3): 433-448.

Vlaeminck, S. E., Geets, J., Vervaeren, H., Boon, N. and Verstraete, W. (2007). "Reactivation of aerobic and anaerobic ammonium oxidizers in OLAND biomass after long-term storage." Applied Microbiology and Biotechnology **74**(6): 1376-1384.

Vlaeminck, S. E., Terada, A., Smets, B. F., De Clippeleir, H., Schaubroeck, T., Bolca, S., Demeestere, L., Mast, J., Boon, N., Carballa, M. and Verstraete, W. (2010). "Aggregate size and architecture determine microbial activity balance for one-stage partial nitritation and anammox." Applied and Environmental Microbiology **76**(3): 900-909.

Vogelsang, C., Gollembiewski, K. and Ostgaard, K. (1999). "Effect of preservation techniques on the regeneration of gel entrapped nitrifying sludge." Water Research **33**(1): 164-168.

Vorobev, A., Jagadevan, S., Jain, S., Anantharaman, K., Dick, G. J., Vuilleumier, S. and Semrau, J. D. (2014). "Genomic and transcriptomic analyses of the facultative methanotroph *Methylocystis* sp. strain SB2 grown on methane or ethanol." Applied and Environmental Microbiology **80**(10): 3044-3052.

Vorobev, A. V., Baani, M., Doronina, N. V., Brady, A. L., Liesack, W., Dunfield, P. F. and Dedysh, S. N. (2011). "*Methyloferula stellata* gen. nov., sp nov., an acidophilic, obligately methanotrophic bacterium that possesses only a soluble methane monooxygenase." International Journal of Systematic and Evolutionary Microbiology **61**: 2456-2463.

Vuilleumier, S., Chistoserdova, L., Lee, M. C., Bringel, F., Lajus, A., Zhou, Y., Gourion, B., Barbe, V., Chang, J., Cruveiller, S., Dossat, C., Gillett, W., Gruffaz, C., Haugen, E., Hourcade, E., Levy, R., Mangenot, S., Muller, E., Nadalig, T., Pagni, M., Penny, C., Peyraud, R., Robinson, D. G., Roche, D., Rouy, Z., Saenampechek, C., Salvignol, G., Vallenet, D., Wu, Z. N., Marx, C. J., Vorholt, J. A., Olson, M. V., Kaul, R., Weissenbach, J., Medigue, C. and Lidstrom, M. E. (2009). "Methylobacterium genome sequences: A reference blueprint to investigate microbial metabolism of C1 compounds from natural and industrial sources." PLOS ONE **4**(5), e5584. doi: 10.1371/journal.pone.0005584.

Wagner, M. (2009). "Single-cell ecophysiology of microbes as revealed by Raman microspectroscopy or secondary ion mass spectrometry imaging." Annual Review of Microbiology **63**: 411-429.

Waite, D. W., Deines, P. and Taylor, M. W. (2013). "Quantifying the impact of storage procedures for faecal bacteriotherapy in the critically endangered New Zealand Parrot, the Kakapo (*Strigops habroptilus*)." Zoo Biol **32**(6): 620-625.

Wang, Q., Garrity, G. M., Tiedje, J. M. and Cole, J. R. (2007). "Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy." Applied and Environmental Microbiology **73**(16): 5261-5267.

Wang, Y., Huang, W. E., Cui, L. and Wagner, M. (2016). "Single cell stable isotope probing in microbiology using Raman microspectroscopy." Current Opinion in Biotechnology **41**: 34-42.

Wang, Z. P., Gu, Q., Deng, F. D., Huang, J. H., Megonigal, J. P., Yu, Q., Lu, X. T., Li, L. H., Chang, S., Zhang, Y. H., Feng, J. C. and Han, X. G. (2016). "Methane emissions from the trunks of living trees on upland soils." The New Phytologist, 10.1111/nph.13909. doi: 10.1111/nph.13909.

Ward, N., Larsen, O., Sakwa, J., Bruseth, L., Khouri, H., Durkin, A. S., Dimitrov, G., Jiang, L. X., Scanlan, D., Kang, K. H., Lewis, M., Nelson, K. E., Methe, B., Wu, M., Heidelberg, J. F., Paulsen, I. T., Fouts, D., Ravel, J., Tettelin, H., Ren, Q. H., Read, T., DeBoy, R. T., Seshadri, R., Salzberg, S. L., Jensen, H. B., Birkeland, N. K., Nelson, W. C., Dodson, R. J., Grindhaug, S. H., Holt, I., Eidhammer, I., Jonasen, I., Vanaken, S., Utterback, T., Feldblyum, T. V., Fraser, C. M., Lillehaug, J. R. and Eisen, J. A. (2004). "Genomic insights into methanotrophy: The

complete genome sequence of *Methylococcus capsulatus* (Bath)." Plos Biology **2**(10): 1616-1628.

Watrous, J., Roach, P., Alexandrov, T., Heath, B. S., Yang, J. Y., Kersten, R. D., van der Voort, M., Pogliano, K., Gross, H., Raaijmakers, J. M., Moore, B. S., Laskin, J., Bandeira, N. and Dorrestein, P. C. (2012). "Mass spectral molecular networking of living microbial colonies." Proceedings of the National Academy of Sciences of the United States of America **109**(26): E1743-E1752.

Watsuji, T. O., Yamamoto, A., Takaki, Y., Ueda, K., Kawagucci, S. and Takai, K. (2014). "Diversity and methane oxidation of active epibiotic methanotrophs on live Shinkaia crosnieri." The ISME Journal **8**(5): 1020-1031.

Wei, X.-M., Su, Y., Zhang, H.-T., Chen, M. and He, R. (2015). "Responses of methanotrophic activity, community and EPS production to CH_4 and O_2 concentrations in waste biocover soils." Waste Management **42**: 118-127.

Wei, Z., Ye, Q., Li, B., Chen, Z. and Huang, Q. (2015). "Removal of dimethyl sulphide in a biotrickling filter under thermophilic conditions." Journal of Bioremediation & Biodegredation **6**(6): 1000320. doi: 10.4172/2155-6199.1000320.

Werner, J. J., Koren, O., Hugenholtz, P., DeSantis, T. Z., Walters, W. A., Caporaso, J. G., Angenent, L. T., Knight, R. and Ley, R. E. (2012). "Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys." The ISME Journal **6**(1): 94-103.

Whittenbury, R., Phillips, K. C. and Wilkinson, J. F. (1970). "Enrichment, isolation and some properties of methane-utilizing bacteria." Journal of General Microbiology **61**: 205-218.

Widder, S., Allen, R. J., Pfeiffer, T., Curtis, T. P., Wiuf, C., Sloan, W. T., Cordero, O. X., Brown, S. P., Momeni, B. and Shou, W. (2016). "Challenges in microbial ecology: Building predictive understanding of community function and dynamics." The ISME Journal, 10.1038/ismej.2016.45.

Wilhelm, D. J., Simbeck, D. R., Karp, A. D. and Dickenson, R. L. (2001). "Syngas production for gas-to-liquids applications: Technologies, issues and outlook." Fuel Processing Technology **71**(1-3): 139-148.

Wilkinson, T. G. and Hamer, G. (1974). "Wall growth in mixed bacterial cultures growing on methane." Biotechnology and Bioengineering **16**(2): 251-260.

Wilkinson, T. G., Topiwala, H. H. and Hamer, G. (1974). "Interactions in a mixed bacterial population growing on methane in continuous culture." Biotechnology and Bioengineering **16**(1): 41-59.

Wilshusen, J. H., Hettiaratchi, J. P. A., De Visscher, A. and Saint-Fort, R. (2004). "Methane oxidation and formation of EPS in compost: Effect of oxygen concentration." Environmental Pollution **129**(2): 305-314.

Wintzingerode, F. v., Göbel, U. B. and Stackebrandt, E. (1997). "Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA

analysis." FEMS Microbiology Reviews **21**(3): 213-229.

Wittebolle, L., Marzorati, M., Clement, L., Balloi, A., Daffonchio, D., Heylen, K., De Vos, P., Verstraete, W. and Boon, N. (2009). "Initial community evenness favours functionality under selective stress." Nature **458**(7238): 623-626.

Wu, M. L., Ettwig, K. F., Jetten, M. S. M., Strous, M., Keltjens, J. T. and van Niftrik, L. (2011). "A new intra-aerobic metabolism in the nitrite-dependent anaerobic methane-oxidizing bacterium *Candidatus* 'Methylomirabilis oxyfera'." Biochemical Society Transactions **39**: 243-248.

Wu, M. L., Wessels, H. J. C. T., Pol, A., Op den Camp, H. J. M., Jetten, M. S. M., van Niftrik, L. and Keltjens, J. T. (2015). "XoxF-Type methanol dehydrogenase from the anaerobic methanotroph "*Candidatus* Methylomirabilis oxyfera"." Applied and Environmental Microbiology **81**(4): 1442-1451.

Yang, S., Matsen, J. B., Konopka, M., Green-Saxena, A., Clubb, J., Sadilek, M., Orphan, V. J., Beck, D. and Kalyuzhnaya, M. G. (2013). "Global molecular analyses of methane metabolism in methanotrophic Alphaproteobacterium, *Methylosinus trichosporium* OB3b. Part II. metabolomics and 13C-labeling study." Frontiers in Microbiology **4**: 70. doi: 10.3389/fmicb.2013.00070.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. and Madden, T. L. (2012). "Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction." BMC Bioinformatics **13**(1), 1-11. doi: 10.1186/1471-2105-13-134.

Ye, R. W., Yao, H., Stead, K., Wang, T., Tao, L., Cheng, Q., Sharpe, P. L., Suh, W., Nagel, E., Arcilla, D., Dragotta, D. and Miller, E. S. (2007). "Construction of the astaxanthin biosynthetic pathway in a methanotrophic bacterium *Methylomonas* sp. strain 16a." Journal of Industrial Microbiology & Biotechnology **34**(4): 289-299.

Yoon, S., Carey, J. N. and Semrau, J. D. (2009). "Feasibility of atmospheric methane removal using methanotrophic biotrickling filters." Applied Microbiology and Biotechnology **83**(5): 949-956.

Yu, Y. H., Ramsay, J. and Ramsay, B. (2009). "Use of allylthiourea to produce soluble methane monooxygenase in the presence of copper." Applied Microbiology and Biotechnology **82**(2): 333-339.

Yusuf, R. O., Noor, Z. Z., Abba, A. H., Hassan, M. A. A. and Din, M. F. M. (2012). "Methane emission by sectors: A comprehensive review of emission sources and mitigation methods." Renewable and Sustainable Energy Reviews **16**(7): 5059-5070.

Zelezniak, A., Andrejev, S., Ponomarova, O., Mende, D. R., Bork, P. and Patil, K. R. (2015). "Metabolic dependencies drive species co-occurrence in diverse microbial communities." Proceedings of the National Academy of Sciences of the United States of America **112**(20): 6449-6454.

Zhang, P. R., Ren, L. H., Zhang, X., Shan, Y. F., Wang, Y., Ji, Y. T., Yin, H. B., Huang, W. E., Xu, J. and Ma, B. (2015). "Raman-

activated cell sorting based on dielectrophoretic single-cell trap and release." Analytical Chemistry **87**(4): 2282-2289.

Zheng, Y., Huang, R., Wang, B. Z., Bodelier, P. L. E. and Jia, Z. J. (2014). "Competitive interactions between methane- and ammonia-oxidizing bacteria modulate carbon and nitrogen cycling in paddy soil." Biogeosciences **11**(12): 3353-3368.

Zhu, J., Wang, Q., Yuan, M., Tan, G.-Y. A., Sun, F., Wang, C., Wu, W. and Lee, P.-H. (2016). "Microbiology and potential applications of aerobic methane oxidation coupled to denitrification (AME-D) process: A review." Water Research **90**: 203-215.

Zhuang, K., Izallalen, M., Mouser, P., Richter, H., Risso, C., Mahadevan, R. and Lovley, D. R. (2011). "Genome-scale dynamic modeling of the competition between *Rhodoferax* and *Geobacter* in anoxic subsurface environments." The ISME Journal **5**(2): 305-316.

CURRICULUM VITAE

CURRICULUM VITAE

Frederiek-Maarten Kerckhof graduated from Ghent University (Belgium) in 2011 with a master in Bioscience Engineering (cellular-and genetic biotechnology). From August 2011 to April 2016, he fulfilled the requirements for a Ph.D at the laboratory for Microbial Ecology and Technology. In his PhD study, he focused on cryopreservation of mixed microbial communities, such as methanotrophic consortia, as well as the interactions and partnerships of methanotrophic bacteria with non-methanotrophic partners. He guided a total of five bachelor and master students during their thesis/bachelor course. Additionally, he followed a variety of specialist courses in the frame of doctoral schools in Ghent University. He was responsible for several practical courses for the Bachelor and Master students of the faculty of Bioscience Engineering of Ghent University focusing on molecular microbial techniques and microbial ecological processes. Additionally, he organized and guided internal courses on statistics, bio-informatics and version control for his collaborators. At the same time, he supported his colleagues with bio-informatics or statistical consultations. These activities led to several journal publications and were presented at different national and international conferences.

PERSONALIA

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EDUCATION

2016	PhD Applied Biological Sciences cellular & genetic biotechnology, Faculty of Bioscience Engineering, Ghent University.
2011	MS Bioscience Engineering: cellular & genetic biotechnology, Ghent University. Summa Cum Laude.
2009	BA Bioscience Engineering: cellular & genetic biotechnology. Cum Laude.
2006	Secondary education: Latin-Mathematics, Onze-Lieve-Vrouwecollege Assebroek (OLVA)

PROFESSIONAL EXPERIENCE

2011-2016	PhD student at the Laboratory for Microbial Ecology and Technology (LabMET) Promotor: Prof. dr. ir. Nico Boon Co-promotor: dr. Kim Heylen

TEACHING EXPERIENCE

2011-2013	Practical exercises molecular microbial techniques (MSc Bioscience engineering)
2013-2015	Practical exercises microbial ecological processes (BSc Bioscience engineering)

Internal training organized for all collaborators within CMET to enhance the quality of research of the group.

-Amplicon NGS analysis: basic training for all collaborators on use of linux command line, mothur and R.

-Statistics and experimental design: introduction to R, proper experimental design and one-way multiple comparisons.

-Version control: use of Git/GitHub for collaborative code editing.

INTERNAL SERVICES

2011-present	Server management of CMETs core computational infrastructure: setup of 3 linux servers, user and software management
2014-2015	Organization of internal research cluster meetings on NGS sequencing and microbial ecology and interactions.
2015	Benchmarking of amplicon sequencing pipelines using mock communities – design of amplicon sequencing SOP.
2016	Setup and management of shiny application server, setup of Pathwaytools server

EXTRA-CURRICULAR ACTIVITIES

1994-2015	Member of Scouts & Gidsen Vlaanderen, Leader of a group of 300 members and 40 leaders, District commissary of the Bruges district with 2500 members and 300 leaders.
2011-now	Contributor to stack Exchange fora (StackOverow, CrossValidated, Ask Ubuntu, TeX) Bio-informatics and statistical consultation

SCIENTIFIC CONTRIBUTIONS

PUBLICATIONS

International peer-reviewed publications (A1 publications)

Allais, L., **Kerckhof, F.-M.,** Verscheure, S., Bracke, K., De Smet, R., Laukens, D., Van den Abbeele, P., et al. (2015). Chronic cigarette smoke exposure induces microbial and inflammatory shifts and mucin changes in the murine gut. (K. Timmis, Ed.) ENVIRONMENTAL MICROBIOLOGY.

Benner, J., De Smet, D., Ho, A., **Kerckhof, F.-M**., Vanhaecke, L., Heylen, K., & Boon, N. (2015). Exploring methane-oxidizing communities for the co-metabolic degradation of organic micropollutants. APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, 99(8), 3609–3618.

Callewaert, C., Van Nevel, S., **Kerckhof, F.-M**., Granitsiotis, M. S., & Boon, N. (2015). Bacterial exchange in household washing machines. FRONTIERS IN MICROBIOLOGY, 6.

Callewaert, C., **Kerckhof, F.-M.,** Granitsiotis, M. S., Van Gele, M., Van de Wiele, T., & Boon, N. (2013). Characterization of Staphylococcus and Corynebacterium clusters in the human axillary region. PLOS ONE, 8(8), e70538.

Callewaert, C., De Maeseneire, E., **Kerckhof, F.-M.,** Verliefde, A., Van de Wiele, T., & Boon, N. (2014). Microbial odor profile of polyester and cotton clothes after a fitness session. APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 80(21), 6611–6619.

De Ryck, T., Grootaert, C., Jaspaert, L., **Kerckhof, F.-M.,** Van Gele, M., De Schrijver, J., Van den Abbeele, P., et al. (2014). Development of an oral mucosa model to study host-microbiome interactions during wound healing. APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, 98(15), 6831–6846.

Ho, A., **Kerckhof, F.-M.,** Luke, C., Reim, A., Krause, S., Boon, N., & Bodelier, P. L. (2013). Conceptualizing functional traits and ecological characteristics of methane-oxidizing bacteria as life strategies. ENVIRONMENTAL MICROBIOLOGY REPORTS, 5(3), 335–345.

Kerckhof, F.-M., Courtens, E., Geirnaert, A., Hoefman, S., Ho, A., Vilchez Vargas, R., Pieper, D. H., et al. (2014). Optimized cryopreservation of mixed microbial communities for conserved functionality and diversity. PLOS ONE, 9(6), e99517.

Marzorati, M., Maignien, L., Verhelst, A., Luta, G., Sinnott, R., **Kerckhof, F.-M.,** Boon, N., Van de Wiele, T. & Possemiers, S. (2013). Barcoded pyrosequencing analysis of the microbial community in a simulator of the human gastrointestinal tract showed a colon region-specific microbiota modulation for two plant-derived polysaccharide blends. ANTONIE VAN LEEUWENHOEK, 103(2), 409–420.

Stock, M., Hoefman, S., **Kerckhof, F.-M.,** Boon, N., De Vos, P., De Baets, B., Heylen, K., & Waegeman, W. (2013). Exploration and prediction of interactions between methanotrophs and heterotrophs. RESEARCH IN MICROBIOLOGY, 164(10), 1045–1054.

Tsilia, V., Uyttendaele, M., **Kerckhof, F.-M.,** Rajkovic, A., Heyndrickx, M., & Van de Wiele, T. (2015). *Bacillus cereus* adhesion to simulated intestinal mucus is determined by its growth on mucin, rather than intestinal environmental parameters. FOODBORNE PATHOGENS AND DISEASE, 12(11), 904–913.

Tsilia, V., **Kerckhof, F.-M**., Rajkovic, A., Heyndrickx, M., & Van de Wiele, T. (2016). *Bacillus cereus* NVH 0500/00 can adhere to mucin but cannot produce enterotoxins during gastrointestinal simulation. APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 82(1), 289–296.

Van den Abbeele, P., Belzer, C., Goossens, M., Kleerebezem, M., De Vos, W. M., Thas, O., De Weirdt, R., **Kerckhof, F.-M**., & Van de Wiele, T. (2013). Butyrate-producing Clostridium cluster XIVa species specifically colonize mucins in an *in vitro* gut model. ISME JOURNAL, 7(5), 949–961.

van der Ha, D., Nachtergaele, L., **Kerckhof, F.-M.,** Rameiyanti, D., Bossier, P., Verstraete, W., & Boon, N. (2012). Conversion of biogas to bioproducts by algae and methane oxidizing bacteria. ENVIRONMENTAL SCIENCE & TECHNOLOGY, 46(24), 13425–13431.

Van Meervenne, E., Van Coillie, E., **Kerckhof, F.-M.,** Devlieghere, F., Herman, L., De Gelder, L., Top, E. M., & Boon, N. (2012). Strain-specific transfer of antibiotic resistance from an environmental plasmid to foodborne pathogens. JOURNAL OF BIOMEDICINE AND BIOTECHNOLOGY.

A2 publications

De Ryck, T., Boterberg, T., **Kerckhof, F.-M.,** De Schrijver, J., Bracke, M., Van De Wiele, T., & Vanhoecke, B. (2015). Effects of irradiation on epithelial wound healing and microbial diversity in an in-vitro oral mucosa model. JOURNAL OF NUCLEAR MEDICINE AND RADIATION THERAPY, 6(2).

A4 publications

Zekker, I., Vlaeminck, S., Bagchi, S., Courtens, E., De Clippeleir, H., **Kerckhof, F.-M.,** & Boon, N. (2012). Selecting nitrifying inocula on different ammonium concentrations. COMMUNICATIONS IN AGRICULTURAL AND APPLIED BIOLOGICAL SCIENCES, 77(1), 275–279.

PROCEEDINGS – ORAL PRESENTATIONS

Kerckhof, F.-M., Vekeman, B., Geirnaert, A., Courtens, E.N.P., Vilchez-Vargas, R., Ho, A., Heylen, K., Boon, N. (2015) Enhancing microbial cryopreservation: From fastidious microbes to mixed communities. CRYOBIOLOGY, 71(3), 549-550. Presented at CRYO2015, the 52nd Annual Meeting of the Society for Cryobiology and International Conference on Regenerative Medicine, 26-29/07/2015, Ostrava, Czech republic.

Allais, L., **Kerckhof, F.-M**., Verscheure, S., Bracke, K., De Smet, R., Laukens, D., Van den Abbeele, P., et al. (2014). The effect of chronic cigarette smoke exposure on the gut microbiome in healthy mice. BSM Annual meeting, Abstracts. Presented at the BSM Annual meeting 2014: Cell signaling in host-microbe interactions, Belgian Society for Food Microbiology (BSFM).

Callewaert, C., **Kerckhof, F.-M**., Van Gele, M., Van de Wiele, T., & Boon, N. (2012). The human axillary environment harbors two microbiome ecotypes. Belgian Society for Cell and Developmental Biology, Meeting abstracts. Presented at the Belgian Society for Cell and Developmental Biology (BSCDB) Fall meeting 2012 : Epidermal cell biology.

Kerckhof, F.-M., Ho, A., De Rudder, C., Heyer, R., Benndorf, D., Heylen, K. & Boon, N. (2016). Happily ever after? How repeated subcultivation inuences a methanotrophic marriage. In Nederlands Tijdschrift voor Medische Microbiologie, volume 24. ISSN 0929-0176.

Scoma, A., Rifai, R. M., Pini, E., Hernandez Sanabria, E., **Kerckhof, F.-M.,** & Boon, N. (2015). Long-chain hydrocarbon degraders from deep-sea. VLIZ Special Publication (Vol. 71, pp. 115–115). Presented at the VLIZ Young Marine Scientists' Day 2015, Oostende, Belgium: Vlaams Instituut voor de Zee (VLIZ).

Tsilia, V., Devos, S., Rajkovic, A., Van de Wiele, T., Heyndrickx, M., **Kerckhof, F.-M.,** & Devreese, B. (2016). To poison or not?: A proteomic approach to quantify enterotoxins produced from *Bacillus cereus*. Advances and Applications in Metaproteomics, Symposium abstracts. Presented at the Symposium on Advances and Applications in Metaproteomics, Magdeburg, Germany: Max Planck Institute.

Van den Abbeele, P., Belzer, C., Goossens, M., Kleerebezem, M., De Vos, W., Thas, O., De Weirdt, R., **Kerckhof, F.-M.,** & Van de Wiele, T. (2012). Specific Clostridium cluster XIVa species drive the mucosal butyrate production. Gut Day Symposium, 14th, Abstracts. Presented at the 14th Gut Day symposium.

PROCEEDINGS – PRESENTED POSTERS

Kerckhof, F.-M., Ho, A., Granitsiotis, M.S., Heylen, K., Boon, N. (2015). Microbial interdependencies in an enriched aerobic mixed methanotrophic community. Presented at FEMS 2015 – 6th conference of European microbiologists, Maastricht, The Netherlands.

Callewaert, C., **Kerckhof, F.-M.,** Van de Wiele, T., & Boon, N. (2012). The bacterial fingerprint of the armpit and its variation in time. COMMUNICATIONS IN AGRICULTURAL AND APPLIED BIOLOGICAL SCIENCES (Vol. 77). Presented at the 17th PhD Symposium on Applied Biological Sciences.

Allais, L., **Kerckhof, F.-M.,** Verscheure, S., Bracke, K., De Smet, R., Laukens, D., Van den Abbeele, P., et al. (2014). The effect of chronic cigarette smoke exposure on gut microbial diversity in healthy mice. Rowett-INRA, 9th Joint symposium, Abstracts. Presented at the 9th Joint symposium Rowett-INRA 2014: Gut microbiology : from sequence to function.

Allais, L., **Kerckhof, F.-M.,** Verschuere, S., Bracke, K., De Smet, R., Laukens, D., De Vos, M., et al. (2013). Chronic cigarette smoke exposure alters the murine gut microbiome. ACTA GASTRO-ENTEROLOGICA BELGICA (Vol. 76). Presented at the 25th Belgian Week of Gastroenterology.

Allais, L., **Kerckhof, F.-M.,** Verschuere, S., Bracke, K., De Smet, R., Laukens, D., De Vos, M., et al. (2013). Chronic cigarette smoke exposure alters the murine gut microbiome. ECCO, 8th Congress, Abstracts (pp. 167–167). Presented at the 8th Congress of ECCO, European Crohn's and Colitis Organisation (ECCO).

Allais, L., **Kerckhof, F.-M.,** Verscheure, S., Bracke, K., De Smet, R., Laukens, D., De Vos, M., et al. (2013). Chronic cigarette smoke exposure alters the murine gut microbiome. Mucosal Immunology, 16th International congress, Abstracts. Presented at the 16th International congress of Mucosal Immunology (ICMI 2013), Society for Mucosal Immunology.

Callewaert, C., **Kerckhof, F.-M.,** Van Keer, T., Plaquet, T., Meunier, M., Bostoen, J., Verhofstadt, L., et al. (2014). Characterisation of the human malodorous axillary microbiome and a novel treatment to obtain a better body odour. JOURNAL OF INVESTIGATIVE DERMATOLOGY (Vol. 134, pp. S78–S78). Presented at the 44th Annual meeting of the European Society for Dermatological Research (ESDR).

Callewaert, C., **Kerckhof, F.-M.,** Granitsiotis, M., Van Gele, M., Van de Wiele, T., & Boon, N. (2012). The human axillary environment harbors 2 microbiome ecotypes. JOURNAL OF INVESTIGATIVE DERMATOLOGY (Vol. 132, pp. S115–S115). Presented at the 42nd Annual meeting of the European Society for Dermatological Research (ESDR).

De Schrijver, J., Volders, P.-J., **Kerckhof, F.-M**., Obbels, D., Verleyen, E., Vyverman, W., De Meyer, T., et al. (2011). PROKAR-SEQ: an analysis and visualization framework for next-generation sequencing based quantification of prokaryotic communities. Benelux Bioinformatics Conference : proceedings of BBC11 (pp. 47–47). Presented at the 6th Benelux Bioinformatics Conference (BBC'11).

Stock, M., Hoefman, S., **Kerckhof, F.-M**., Boon, N., De Vos, P., Heylen, K., De Baets, B. & Waegeman, W. (2012). A kernel-based model to predict interaction between methanotrophic and heterotrophic bacteria. Communications in agricultural and applied biological sciences, 78(1):55-60.

Van den Abbeele, P., Belzer, C., Goossens, M., Kleerebezem, M., De Vos, W., Thas, O., De Weirdt, R., **Kerckhof, F.-M**., & Van de Wiele, T. (2012). Specific butyrate-producing Clostridium cluster XIVa species colonize the mucosal environment of a novel in vitro gut model. Gut Microbiology, 8th Joint symposium INRA-RRI, Abstracts. Presented at the 8th Joint symposium INRA-RRI on Gut Microbiology : Gut microbiota : friend or foe?

Van Meervenne, E., Van Coillie, E., **Kerckhof, F.-M**., Devlieghere, F., Herman, L., De Gelder, L., Top, E., et al. (2012). An environmental multiresistance plasmid can be transferred to foodborne pathogens. FoodMicro 2012, Abstracts. Presented at the 23rd International ICFMH symposium FoodMicro 2012 : Global issues in food microbiology.

Van Meervenne, E., Van Coillie, E., **Kerckhof, F.-M**., Devlieghere, F., Herman, L., De Gelder, L., Top, E., et al. (2012). An environmental multiresistance plasmid can be transferred to foodborne pathogens.

Microbial Ecology, 14th International symposium, Abstracts. Presented at the 14th International symposium on Microbial Ecology (ISME-14) : The power of the small.

DANKWOORD

DANKWOORD

Scientific research is challenging, and more than often you don't find what you were looking for. But maybe, just maybe, that's when you discover something interesting.

Ik herinner me het nog levendig. Als kersverse 2e master Bio-ingenieurswetenschappen, cel-en-gen biotechnologie was ik de enige op mijn ganse jaar die voor een "milietechnologische" thesis koos. CMET (toen nog LabMET) had mijn interesse veroverd met een thesisonderwerp rond microbial fuel cells (MFCs, in het pre-prof. Rabaey tijdperk… voor BES binnen CMET "hip" waren). Mijn jaargenoten kozen bijna uitsluitend onderwerpen binnen rode- en groene biotech maar ik was door de bacteriële wereld gebeten. Het concept van bacteriën die –al dan niet rechtstreeks – een vaste stof (een electrode) als elektron- donor of acceptor zouden kunnen gebruiken daar ging ik mij eens op toeleggen. En ik zou een bijdrage kunnen leveren (op 1 jaar tijd) aan het wetenschappelijk onderzoek in dit onderwerp. Dat ene jaar heb ik waarschijnlijk elk lab en elk apparaat dat CMET toen rijk was gezien. Onder begeleiding van eerst Jan Schouppe, toen niemand en uiteindelijk Jan Arends die zich over mij ontfermde (terwijl hij zelf al 4 mensen moest begeleiden) heb ik duizend-en-één dingen geprobeerd. Ik had de passie voor research te pakken. Dit wou ik blijven doen. Op het eind van de rit had ik veel geleerd, maar ik had niet het gevoel dat ik een echte bijdrage had kunnen leveren aan het veld.

Én toch. Toch nam prof. Boon me mee om een pint gaan drinken na mijn verdediging in de Koepuur om over mijn toekomst te praten. Hij vertelde me over dit interessante GOA project over "duurzame methanotrofen". Ik vond dit fantastisch, en vertelde dat ik hier zeker in mee wou gaan. Toen hij me vroeg wanneer ik kon beginnen, zei ik meteen "augustus", maar misschien had ik mezelf toen beter een beetje vakantie gegund. Want sinds die augustus in 2011, nu bijna 5 jaar geleden, heb ik geen moment stilgezeten (of deftig geslapen).

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Naast Kim mocht ik ook prof De Vos, Sven en Bram van het LM-UGent ontmoeten binnen het GOA project. Jullie manier van werken heeft me veel geleerd dat in CMET niet altijd gekend was. Dankjewel voor jullie steun, input en de vele malen dat ik pure MOB culturen bij jullie mocht komen ophalen. David, ook aan jou een enorme merci om mij in te wijden in de wondere wereld van de MOB (ik heb je originele mengcultuur nog altijd op de shaker). Adrian, also many thanks to you. I really enjoyed our collaborations when you were still in the office, but now it is just as if you've never left when we meet each other on conferences and online. Giovanni, thank you too for the methanotrophic and non-methanotrophic fun we had together. Specifiek wil ik graag nog even jou toch bedanken Bram. We gingen samen naar de Gordon C1 en we waren er voor elkaars laatste loodjes. Samen waren we de laatste methanotrofe cowboys binnen de UGent. Bedankt voor jouw gelijkgezindheid en input!

Working at CMET could be hell. There's a lot of people, a lot of pressure and not always enough equipment for all of us. And while I enjoyed the peace and quiet (and not having to queue for any equipment) when working nights, working at CMET (during day) is made a true delight by all of the CMET colleagues. We've really got a good thing going guys. All of us. Though I would like to thank some of you in specific, I am grateful to all of you. Nevertheless, first I would like to thank my office, Office 74: Adrian, Baharak, Chris, Emma,

Giovanni, Lai, Nicole, Racha, Ralph and Yusuf… since CMET became my second home you guys were my second family. We've created many good memories in the office. Thank you for that. Also outside "the best office in the world" I'd like to send my gratitude to: Amanda, Annelies, Antonin, Benjamin, Cristina², Curro, Dries, Eleni, Emilie, Emmeh, Eva, Flo, Francis, Greet, Hugo, Jan, Jana, Jessica, Jianyun, Jo², Joachim, Joeri, Karen, [KT]immeh, Mar-Mar, Mike, Nikki, Ramiro, Renée, Robin, Ruben, Sarah, Siska, Sofie, Sue, Thijs & Tom: one way or another you made CMET a better place for me. To the people I forgot in the list above: no worries, it's not intended to be exhaustive but I am currently exhausted, which may affect my memories.

Dear CMET collaborators, I know I was the annoying guy always asking questions about methodology and statistics during the seminar, though I want to thank many of you (and specifically Chris) for your confidence in my biostatistics and bioinformatics work and allowing me to co-author on the resulting publications. It allowed me to keep an open look and keep on investigating many domains of science from the human microbiome to high-pressure oil spill degraders. Lois, thanks for introducing me to mothur and Kim, thanks for all the joint work on the Illumina amplicon SOP. I loved trying to make more commits than you on github, it was always a nice race. Also outside of CMET I've had the pleasure to collaborate with some truly remarkable scientists. Michael, Ευχαριστώ για την συνεργασία σου! I know you've had a bumpy ride as well and I hope better days are coming your way, I'll contribute wherever I can and look forward to our future collaborations. Robert & Liesbeth, thanks for introducing me to the world of metaproteomics and IBD resprectively. Michiel, it was also a pleasure working with you! First during the Machine Learning course and afterwards on the experiment where you and Sven combined MOB and non-MOB.

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Your cynical humor knows no bounds.
Tim, O scientific rambler!
Our long discussions (in whatever degree of sobriety) always lead to scientific insights.
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Gent, June 4th, 2016
Frederiek-Maarten Kerckhof